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Dedicated to
my wife, Gretta,
my daughters, Heather and Amanda,
and my parents

FOREWORD

It has been just over 10 years since the technique of high-performance liquid chromatography took its first hesitant steps into the clinical laboratory. Since that time, it has deservedly become the technique of choice for many applications in therapeutic drug monitoring and toxicology. Modern liquid chromatography (LC) has made it possible for laboratorians to routinely measure many previously elusive analytes. Just as importantly, it has allowed the determination of highly polar, conjugated metabolites of therapeutic agents, the concentrations of which may be predictive of certain metabolic diseases or drug overdose. This latter capability of LC is not shared by currently available immunoassays. There is promise for the future: improved sensitivity, more specific detectors, and partial or full automation are now becoming available or are on the horizon.

This volume is an excellent representation of the many areas of active investigation in the clinical application of LC to drug analysis. Its editor and contributors are to be congratulated for their straightforward and pragmatic approach to this instrumental technique. I believe that their effort will promote and encourage the adoption of LC as a practical analytical technique by numerous clinical laboratories throughout the world.

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PREFACE

Therapeutic drug monitoring (TDM), since its emergence in the 70s, has been established as a well-accepted subspecialty of clinical medicine. Undoubtedly, laboratory measurements of TDM and toxicology have been greatly enhanced by the advances in instrumentations such as immunoassay and chromatography. Liquid chromatography (LC), in particular, has played a very active and vital role, by providing fast, specific, cost-effective, and in selected cases (such as antidepressants, antihypertensives, and immunosuppressives), the preferred or only mode of analysis for drugs and metabolites, often before the immunoassay. Indeed, for comparison studies, the LC assay is often chosen as the reference method. In spite of numerous books, articles, and workshops dealing with various aspects of TDM and toxicology, a comprehensive and dedicated treatise of LC for these areas is still lacking. Furthermore, with the present emphasis on the new health-care economics, such as the Diagnostic Related Group (DRG) reimbursement policy, laboratories might want to reexamine the choice of instrumentation and methods. It is in light of these considerations that this book was conceived and finalized. The approach is to present a current account of the various LC instrumentations for TDM and toxicology drug assays, and to review the clinical pharmacology of major classes of drugs and their LC analyses. Each chapter is based upon its contributor's personal experience. The book should be useful to practitioners such as laboratory directors, toxicologists, and clinicians, and to interested nonpractitioners such as analytical chemists, immunologists, and instrument manufacturers.

The first part includes chapters on the principles of TDM, sampling techniques, and various instrumentation topics, such as the computer,

mass spectrometry, fluorescence, and electrochemical detection. The second part consists of chapters on six major classes of drugs, beginning with a review of their clinical pharmacology and LC analyses, followed by recommended LC procedures. Emphasis is placed on some of the recently introduced drugs and their LC analyses. The third part consists of chapters on medicolegal guidelines, LC analyses of miscellaneous groups of drugs, and various laboratory management considerations. It is hoped that through this updated and coordinated treatment, the roles of LC might be better defined to meet the present needs of the laboratory for TDM and toxicology drug measurements.

The editor gratefully acknowledges the effort of the contributors. He is indebted to his chairman, F. William Sunderman, Jr., M.D., for his encouragement and support; and his former mentor, Dr. Hans J. Ache, for introducing liquid chromatography to his graduate research. During the initial planning of this book, valuable suggestions were solicited from the following persons: Drs. Richard P. Spencer, Jack Cazes, Charles Pippenger, R. P. W. Scott, B. L. Karger, L. R. Snyder, and John Dolan. He also wishes to thank Drs. Dennis Hill, Carlos Santiago, Ralph Rodriquez, Mitchell Gandleman, and Nemat Marzouk for reviewing the manuscripts; and Mary Gagnon who undertook the laborious and important tasks of typing the manuscripts and correspondence. His family's understanding and support has inspired and enhanced the undertaking and completion of this project.

Steven H. Y. Wong

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1

INTRODUCTION

STEVEN H. Y. WONG / *University of Connecticut School of Medicine, Farmington, Connecticut*

Therapeutic drug monitoring (TDM) has developed into a well-established subspecialty within the clinical laboratory. It deals with the optimization of the clinical response, as guided by the drug concentration in biological fluid such as plasma or serum. The rationale and the principles for TDM have been extensively reviewed [1-5] and updated in Chap. 2 by Pippenger. Toxicology deals with the identification/quantitation of known or unknown drugs and chemicals [6-9]. Traditionally, some of the modern TDM functions have been regarded as toxicology. Depending on the drug concentration and patient history (such as overdose), a TDM or toxicology measurement may be performed by the same procedure.

The evolution of TDM resulted from the increasing abundance of pharmacokinetic data [10], and from the introduction and/or improvement of instrumentations such as chromatography and immunoassay. With these instrumentations, laboratory personnel can measure the plasma concentration of drugs and metabolites within a reasonable amount of time (usually 1-4 hr), with predictable and acceptable precision (relative standard deviations are usually less than 10%) [11]. Among the various modes of chromatography used for TDM and toxicology, both gas and liquid chromatography have played vital roles. Applications of LC for drug measurement dated back to the early seventies with the advent of LC for drug identification and for pharmacokinetic studies [12]. Liquid chromatography, herein encompassing the following terms, "high-performance liquid chromatography (HPLC)," "high-pressure liquid chromatography (HPLC)," and "high-speed liquid chromatography," has already surpassed gas chromatographic applications for certain areas of TDM such as antidepressant monitoring,

according to a recent survey result of the College of American Pathologists, as shown by Table 1 for the measurement of nortriptyline [13]. Advantages of LC include its selectivity and sensitivity as a result of variety of separation and detection modes. Thus LC has enhanced TDM and toxicology as much as it has other areas of the biomedical and biochemical sciences. But perhaps the difference in regard to LC is the high frequency of daily routine LC clinical analysis—a testimonial to the remarkable state of the instrumentation and column technology.

In carrying out TDM/toxicology measurements, there are two primary considerations—personnel and instrumentation. A dedicated number of personnel (four to five technologists) would enhance the operation with the objective of becoming an active and integral part in the patient health care system. In view of the vast number of drugs and the increasing number of newly introduced drugs on the market, the technologists would have to gain a working knowledge of clinical pharmacology through meetings, discussions, and workshops. Then the personnel would be more proficient towards interpreting drug levels as well as detecting abnormal results or laboratory errors. The dosage regime and the clinical status of the patients would guide the laboratory personnel as to what to anticipate, and therefore to recommend the appropriate drug measurement/toxicology test. The recommendations would include sampling time, types of specimens, and others. Based on the above considerations, it is inherently difficult to train a large laboratory staff to be proficient in various aspects of clinical pharmacology and measurement.

For the measurement or identification of drugs and chemicals, the instrumentation ranges from the simple flame photometer used to measure lithium, to automated equipment such as the Automated Clinical Analyzer (Dupont, Wilmington, Delaware) used for Enzyme Multiplied Immunoassay Technique (EMIT) (Syva, Palo Alto, California), and TDX, for Fluorescence Polarization Immunoassay (FPIA) (Abbott, North Chicago, Illinois), to the more demanding chromatography. The immunoassay technology and its instrumentation are much more adaptable to automation and thus require less involvement of personnel. However, the chromatographic procedures usually require extraction and analysis performed with close personal attention. Depending upon the ability of the personnel, TDM/toxicology chromatographic equipment may range from simple TLC and basic gas or liquid chromatographs, to the more sophisticated chromatographs with various data handling capability such as GC-MS and its associated data bank. In order to utilize these instruments, it will be more efficient to train a dedicated group of technologists.

Among the whole range of instrumentation used by clinical laboratories for drug measurement, chromatography has always played a vital role. From the survey results of the College of American Pathologists [14], phenobarbital measurement (Table 2) is still performed by

Table 1 CAP Survey Results for the Measurement of Nortriptyline (ng/ml)

Specimen/method	No. labs	Mean	S.D.	C.V.	Median	Low value	High value
Specimen Z-5	Target value 100.0						
Gas-liquid chromatography							
Underivatized	18	—	—	—	90	49	151
High-performance liquid chrom.							
Solvent extraction	42	98.7	37.2	37.7	—	—	—
All HPLC results	49	97.8	35.5	36.3	—	—	—
All methods/all results	74	102.6	43.0	42.0	—	—	—
Specimen Z-6	Target value 1200.0						
Gas-liquid chromatography							
Underivatized	18	—	—	—	1243	207	1094
High-performance liquid chrom.							
Solvent extraction	41	1199.8	201.5	16.8	—	—	—
All HPLC results	48	1175.4	170.3	14.5	—	—	—
All methods/all results	72	1230.9	237.2	19.3	—	—	—

Source: Ref. 13.

Table 2 CAP Survey Results for the Measurement of Phenobarbital (mcg/ml)

Specimen/method	No. labs	Mean	S.D.	C.V.	Median	Low value	High value
Specimen Z-2	Target value 2.0						
The results listed below were reported as "Equal To" values.							
Photometry							
Spectrophotometry/colorimetry	11	—	—	—	2.0	1.3	6.0
All photometry results	12	—	—	—	2.0	1.3	6.0
Gas-liquid chromatography							
Derivatized	11	—	—	—	2.0	0.0	2.4
Underivatized	14	—	—	—	2.3	1.2	6.1
All GLC results	24	2.12	0.95	44.7	—	—	—
High-performance liquid chrom.							
Protein precipitation	21	2.45	0.76	31.2	—	—	—
Solvent extraction	21	2.30	0.63	27.6	—	—	—
All HPLC results	45	2.38	0.69	28.8	—	—	—
Enzyme immunoassay							
Syva	105	2.40	0.88	26.6	—	—	—
Other	19	—	—	—	2.2	1.0	19.5

Fluorescent immunoassay							
Ames	18	—	—	—	2.0	0.7	2.5
Fluorescence polarization imm.							
Abbott	84	1.44	0.30	21.0	—	—	—
Immunonephelometry/immunoturbid.							
Beckman	65	3.88	1.22	31.4	—	—	—
Radioimmunoassay							
Clinical assays	13	—	—	—	2.5	1.7	3.3
All immunoassay results	307	2.34	1.02	43.6	—	—	—
All methods/all results	401	2.34	1.01	43.1	—	—	—

The results listed below were reported as "Less Than" values.

	No. labs				Mode	Low value	High value
Photometry							
Spectrophotometry/colorimetry	36	—	—	—	5.0	2.0	5.0
Enzyme immunoassay							
Syva	418	—	—	—	5.0	1.5	5.0
Other	118	—	—	—	5.0	3.0	5.0
Fluorescence polarization imm.							
Abbott	31	—	—	—	5.0	0.5	5.0
Immunonephelometry/immunoturbid.							
Beckman	21	—	—	—	8.0	3.8	8.0

Source: Ref. 14.

a large array of methods, ranging from the traditional, nonspecific colorimetric procedures used by several laboratories, to the more modern methods of immunoassay and chromatography. To monitor a drug such as phenobarbital, which may be coadministered with other antiepileptics such as phenytoin or primidone, chromatography may be the preferred approach because of its low cost and the possibility of simultaneous, multidrug measurement. However, the popular choice is the easily performed immunoassay such as EMIT of FPIA. Further, the choice also depends on the interest and the expertise of the laboratory director. It is fair to conclude that for drug measurement in the mg/L range, chromatography is not as popular as immunoassay. However, for measurement in μg/L range, exemplified by nortriptyline in Table 1 [13], chromatography is the only method. Furthermore, liquid chromatography is preferably used. It may be fair to speculate that LC will be used increasingly over GC to measure drugs in low concentration. Generally, liquid chromatographic procedures require fewer extraction steps as compared to those of gas chromatography. Precision and accuracy are thus enhanced. On the other hand, for analyzing volatile and UV-transparent drugs, gas chromatography would be the method of choice, due to the absence of an established universal mass detector, such as the flame ionization detector, in liquid chromatography. In summary, both liquid and gas chromatography are vitally important to drug monitoring/toxicology laboratories. Depending upon the property of the drug, the availability of instrumentation, and the experience of the analyst, the selection of the procedure would be determined accordingly. Later on, in Chap. 3, a more detailed discussion will be devoted to each mode of instrumentation. A universal guide for selection would be *Simplicity*. A simple procedure would typically include a two- or three-step extraction, followed by isocratic, reversed-phase separation with detection by using a fixed-wavelength detector. Comparing this to the other extreme, a multiple-step (i.e., more than three steps) extraction is followed by a gradient elution with detection by a fluorescence detector. Inherently, the former procedure would most likely offer reproducible and acceptable precision. The more complex procedure would, of course, be more susceptible to instrumentation variations and human errors. Since clinical assays are usually performed by several lab technologists with different levels of chromatographic expertise, the need for simplicity is therefore more apparent. Furthermore, distinction should be drawn between an assay for pharmaceutical analysis where the matrix is usually simple (such as other formulation chemicals), and a clinical drug assay. The latter procedure requires reasonable precision over an extended period of time, typically from 3 to 12 months. Thus a good clinical assay will be one that is time proven, with acceptable within-run and day-to-day relative standard deviation. By carefully evaluating precision and

other factors such as drug and column stability, the assay will most likely remain trouble-free.

The prupose of this book is to present the current status of LC for TDM and toxicology for clinical laboratory directors, supervisors, analytical chemists, and biochemists. In achieving this goal, the book may be readily divided into the three major parts. First, the fundamentals, the instrumentation, sample handling, and treatment are discussed to present a state-of-the-art overview. Second, the clinical pharmacology of major classes of drugs is outlined with insightful discussions of their LC analyses. Third, the medicolegal guidelines and various management considerations are reviewed.

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I

FUNDAMENTALS AND CURRENT CLINICAL INSTRUMENTATION

2

PRINCIPLES OF THERAPEUTIC DRUG MONITORING

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I. INTRODUCTION

Therapeutic drug monitoring is a multidisciplinary field encompassing, among other specialities, clinical pharmacology, clinical pathology, clinical chemistry, toxicology, analytical chemistry, and medicine. In the past, interdisciplinary scientific communication has often suffered from the fact that journals tend to be narrowly discipline-oriented rather than subject-oriented. Yet workers today require broad-based knowledge from a wide variety of subject areas in order to best serve the patient and the clinician.

Both clinicians and pharmacologists have had a long-standing interest in establishing why a fixed drug dosage is therapeutically effective in some individuals but not in others. For years appropriate dosage regimens of drugs were established only by empirical trial-and-error approaches. Modern analytical techniques have provided an alternative. The resulting ability to correlate serum or plasma drug concentrations by inference tissue concentrations, with the observed clinical effect of a given agent has provided new insights into the entire field of therapeutics. Historically, the measurement of serum concentrations was one of the functions of the clinical pharmacology laboratory, but the increasing demand for such measurements to be performed routinely has exceeded the capacity of many laboratories, and has necessitated the establishment of special sections within many clinical chemistry laboratories for processing large numbers of patient specimens. Over the last decade, the demand for this procedure has increased geometrically to the point where therapeutic drug monitoring has evolved into a new scientific discipline [1-10].

Today, utilizing new analytical techniques, we have begun to understand the interrelationships between drug dose and pharmacological effect. We now know that for many drugs the desired pharmacological effect is achieved only after a specific plasma concentration is achieved and that there is usually an optimal plasma concentration range for successful drug therapy. Above this range, patients may begin to experience undesirable drug side effects. Below this range patients may fail to achieve the desired relief from the disease or symptom for which they are receiving therapy. Rapid advances in clinical pharmacology over the past decade are directly attributed to TDM; the availability of TDM, in turn, is directly related to the rapid advancement in technology associated with the quantitation of drug compounds.

A. History of TDM

TDM has been associated with clinical laboratory medicine since the work of Wuth in 1927 [11] demonstrated the value of monitoring serum bromide concentrations to differentiate bromide-induced psychotic behavior from psychotic behavior attributable to other organic causes. During World War II, the search for antimalarial compounds resulted in improved analytical instrumentation, improved techniques for drug quantitation, and new insights into the relationship between drug concentration and therapeutic effectiveness. The first studies correlating plasma drug concentrations with their therapeutic efficacy were published in the late fifties and early sixties.

Not until the late 1960s did TDM become widespread. Gas-liquid chromatography (GLC) represented a major breakthough because it provided a method of rapidly separating and quantitating individual drugs within a given class. Gas-liquid chromatographic techniques were further refined and improved so that by the early 1970s GLC analysis of various therapeutically monitored agents was performed routinely in many clinical chemistry laboratories. One of the major disadvantages of GLC was the complexity of the instrumentation, which necessitated a highly trained and skilled analyst. More recent advances in the development of the nitrogen-phosphorus detector and capillary columns have increased the sensitivity of the instruments to such an extent that drug analyses can be performed routinely on micro volumes of plasma.

The development of radioimmunoassay techniques also permitted quantitation of drug concentrations in microvolumes of serum. Unfortunately, however, the complexity of the technique as well as the lack of radioimmunoassays for a wide variety of drugs prevented its widespread adoption for routine drug monitoring [12].

Making TDM available to all laboratories and physicians required simple technology that could be performed by a technician without special training or instrumentation. This was achieved with the de-

velopment of the homogeneous enzyme immunoassay system (EMIT), which is capable of performing assays on less than 40 μl of serum. The major advantages of the system are its microcapability and accuracy, and the rapidity and ease of operation of the assays. More recently, substrate-labeled fluorescent immunoassays (SLFIA) and fluorescence polarization immunoassays (FPI) for the rapid quantitation of drugs have become available [13].

A large number of drugs exist for which antibodies are not available, but which must be therapeutically monitored. The most promising and practical method of monitoring these agents is by high-pressure liquid chromatography (HPLC). Within the last 9 years, the development of HPLC has provided laboratories with a system having the same advantages as the homogeneous enzyme immunoassay system: it is capable of processing microsamples (100 μl), it is rapid and specific, and the instrumentation is relatively simple to operate. In addition, HPLC can be adapted to quantitate simultaneously a large variety of drugs as well as their active metabolites. High-pressure liquid chromatography permits simultaneous drug analysis and is a valuable tool for establishing correlations between drug and drug metabolite concentrations in biological fluids [14-15].

B. General Clinical Applications of TDM

As with any new laboratory discipline, TDM is not a panacea which will solve all problems associated with drug therapy. There are specific clinical applications just as there are situations where will probably serve no useful purpose. TDM is most applicable when the drug in question has a narrow therapeutic range, is used chronically, has potentially toxic side effects if overdosed, and has minimal therapeutic effects if underdosed. Both clinical and molecular studies of the pharmacological profiles of a wide variety of drugs have demonstrated that a much better correlation exists between the observed clinical effects of a drug and its plasma concentration than that observed between the clinical effect and total daily drug dosage. With this in mind, TDM can be utilized to [16-20]:

1. Recognize noncompliance. Many patients, in particular those who have chronic disease requiring therapy over a prolonged period of time, tend not to take their medications as prescribed. Moreover, patients with a chronic disease that does not chronically cause pain or other unusual discomfort (for example, the epilepsies, asthma or hypertension) may easily neglect to take their medicine. The end result of such noncompliance is an exacerbation of the existing disorder at some time in the future. Studies have clearly demonstrated that noncompliance is a major factor in treatment failures.

2. Compensate appropriately for individual variations in drug utilization patterns. In any population of individuals, a drug dosage based solely on body weight results in a fixed steady-state serum con-

centration. If the plasma concentrations following a specific dosage are analyzed in a large patient population, the distribution of drug levels will be gaussian. The vast majority of patients will have levels within the range expected from a dosage based on body weight. But patients who are genetically either "fast" or "slow" drug metabolizers will have levels at the extreme ends of the curve. Fast drug metabolizers require significantly higher doses to achieve the same plasma concentrations and consequently the desired therapeutic effect. Slow drug metabolizers become intoxicated and experience side effects from standard therapeutic doses of the drugs, and therefore will be maintained at optimal drug levels on dosages well below the standard regimen.

3. Compensate for altered drug utilization associated with various disease states. Patients on long-term drug therapy may become acutely ill and need additional therapeutic agents. Drug interactions may then cause these patients to respond in an unexpected manner to a fixed dosage of medication. Acute or chronic uremia can dramatically decrease the elimination of a drug that is primarily dependent on urinary excretion, and renal failure can alter the protein-binding characteristics of many drugs to albumin. In both situations the ratio of free drug to total drug may increase to the point at which free drug concentrations are high enough to produce a clinically evident drug response, although the total serum drug concentrations are usually within optimal therapeutic range. Moreover, free drug levels can rise into the toxic range while total drug concentrations remain within or even below the usual ranges.

Hepatic disease can extensively alter a given therapeutic response by impairing a patient's ability to metabolize drugs. Most drugs depend on liver detoxification for conversion to water-soluble products, which are easily eliminated from the body. Thus, a precipitous rise in parent drug concentrations can occur as the unmetabolized drug, which normally would have been eliminated from the system, accumulates.

4. Adjust therapeutic drug regimens to compensate for changing physiological states. Normal alternations in physiological state also change drug-utilization patterns. TDM is crucial to successful adjustment of dosage regimen in pregnancy, puberty, and old age.

Recent studies have shown that decreased drug absorption during pregnancy is associated with a decrease in serum phenytoin concentration and exacerbation of seizures in epileptic gravidas. The use of TDM from the onset of pregnancy, with appropriate dosage regulation to maintain therapeutic drug concentrations, significantly decreases the number of seizures that occur, thus decreasing potential harm to the fetus.

The normal process of maturation involves a large number of physiological changes that can dramatically alter drug utilization. Complex changes in drug utilization patterns occur in the weeks following birth. Older children utilize drugs at a faster rate than adults and therefore require almost twice as much drug on a body weight

basis as adults to achieve the same therapeutic drug concentration. As a child enters puberty, drug utilization rapdily changes, to the extent that by early pubescence the converion to adult patterns is complete. These changes usually occur between the ages of 10 and 13, appearing earlier in girls than in boys. Chronic medication must be administered carefully, with frequent blood level determinations, when treating early pubescent children. Failure to adjust the child's therapeutic regimen to compensate for the associated physiological changes may result in exposure to unnecessary and prolonged drug toxicity, with its attendant sequelae.

As the maturation process continues and the efficiency of normal physiological functions decreases, so does the ability to bind drugs to plasma protein. Geriatric patients often exhibit reduced rates of drug elimination, thereby requiring reduced drug dosages. Geriatric patients may have total drug plasma concentrations within the optimal therapeutic range, but elevated free drug concentrations that can produce adverse side effects. The clinical signs of drug intoxication in the elderly often present clinically as lethargy and confusion, and TDM provides a means of distinguishing drug-induced confusion from organic deterioration.

5. Identify the baseline concentrations associated with an optimal therapeutic regimen. After a patient has undergone a strenuous work-up to define an appropriate therapeutic regimen, the physician can establish a baseline drug concentration at which the patient responds well. Should the patient return in the future uncontrolled, the physician can rapidly document whether the patient has been compliant, or whether a new disease state has altered the pharmacological response to the drug.

Numerous factors, including individual differences in drug metabolism and excretion, age, sex, patient compliance, disease, and drug interactions (particularly during multiple-drug therapy) regulate the disposition pattern of a drug within an individual patient. The rate of drug disposition, in turn, regulates the amount of drug available to interact with a receptor. The therapeutic response observed in a given patient is dependent on the sum of all these processes and is directly related to the drug concentration in that particular patient. Interactions between all the potential factors influencing drug disposition account for the broad interpatient variability in plasma concentrations following either single- or multiple-drug doses. Individual patient response to a given drug dose, however, remains constant because the factors which can alter drug utilization within the individual are relatively fixed.

Generally, interindividual variations of response, as demonstrated by the clinical response of a large population to a fixed drug dose, are more of a reflection of the relationship between total daily dose and plasma concentration than they are of the relationship between plasma concentration and the intensity of response. In other words, the

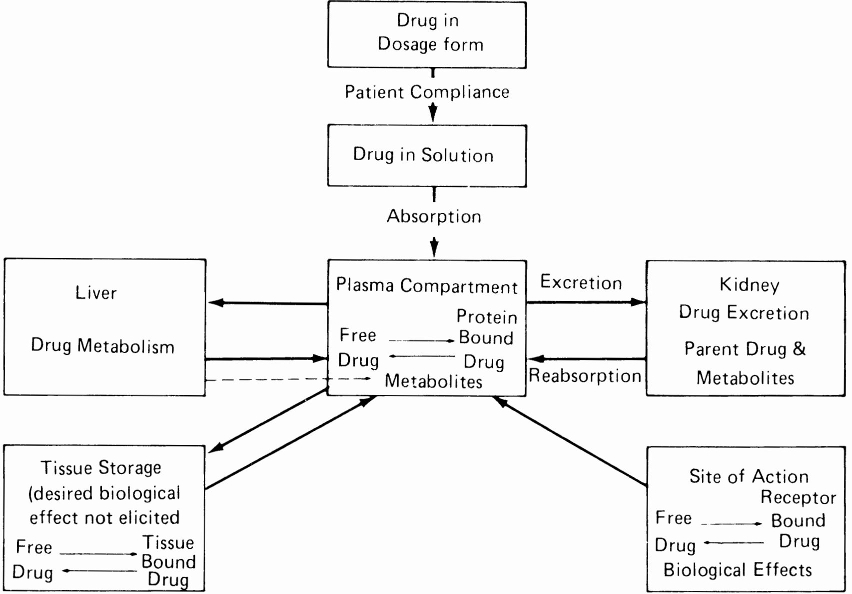
probability of achieving a given plasma concentration from a given drug does is much less than the probability of obtaining a specific biological effect from a given plasma concentration. This is why drugs administered at fixed doses produce marked variations within a population in the observed therapeutic response: When an average or standard drug dosage is administered to a large patient population, the desired therapeutic effect will be achieved in some patients, while no therapeutic effect will occur in others, and toxicity, usually associated with drug overdosage, will be evident in still others.

II. PHARMACODYNAMICS: SITE AND MECHANISM OF DRUG ACTION

The biological effect achieved following a given drug dose is a direct consequence of the formation of reversible bonds between the drug and tissue receptors controlling a particular response. For most drugs, the intensity and duration of a given pharmacological effect is proportional to the drug concentration at the receptor site. The exact mechanism of receptor interactions, however, remains unclear. In order for a drug to exert the desired biological effect, it must reach and interact with the receptors regulating a specific response. In addition, disease, age, sex, compliance, drug interactions, and individual differences in drug metabolism and excretion contribute to interpatient response differences. Figure 1 schematically depicts the factors which can alter the concentration of drugs, ultimately achieved and maintained at a given receptor site. The titration of drug dosage using TDM is the most precise method for achieving therapeutic plasma concentrations, thus indirectly influencing the concentration at receptor sites (see below) and compensating for these interindividual variations in response [3,16].

Every drug acts to produce a change in some known physiological function or process. Any drug may increase, decrease, or return to normal the physiological function of tissues, organs, or physiological systems. The biological effect observed following administration of a drug is the sum of the processes by which that drug creates changes in some physiological or biochemical process. Such effects can be measured and expressed only in terms of alteration of a specific function or process. A change in function due to a drug's pharmacodynamic activity may return the function or physiological process from an abnormal to a normal level of activity. Or, it may prevent deviation from the normal physiological state of a given system.

For most drugs, the intensity of a pharmacological effect tends to be proportional to the drug present in extracellular fluid which can enter tissues and interact with specific receptors to elicit a biological effect. For example, antiepileptic drugs are believed to prevent seizures by binding to neutral membranes or altering neurotransmitter



Absorption

Drug must be formulated in a manner which assures bioavailability for absorption.

Metabolism

Drug converted to a more soluble compound which may be biologically active or inactive. Metabolism can also occur in other tissues.

Excretion

Usually more water soluble drug metabolites are excreted in urine. Also drug excretion can occur via bile, feces, saliva and expired air.

Tissue Storage

Distribution of drug to sites where the desired biological effect is not elicited. Undesirable effects may be elicited by drug interaction with a specific physiological system.

Site of Action

Free drug binds to receptor to elicit a biological effect (response). Number & type of receptors to which drug is bound determines the intensity and duration of the desired and undesired effects.

Figure 1 Factors which regulate tissue drug concentrations. Reproduced from the Syva Monitor with permission.

release. Alteration of these functions is thought to stabilize neutronal membranes against the excessive electrical activity that is responsible for precipitating a clinical seizure.

Figure 2 is a schematic representation of drug distribution following absorption between the plasma and various tissue compartments. Drug concentration in extracellular water is in equilibrium with the drug concentration in plasma water. The latter, assessed in the TDM laboratory by measuring the free drug concentration, is an indirect measure of drug concentration at the *site of action*, which may be defined as the site at which (a) a given drug acts to initiate the events which lead to a specific biological effect, and (b) a drug's biological effect may be elicited by direct interaction with a receptor that controls a specific function, or by alteration of the physiological process which regulates that specific function.

Since many drugs are partially bound to plasma proteins, an equilibrium exists between the concentrations of protein-bound drug and of free drug in plasma water. Only free drug is capable of crossing the various lipoprotein membranes that surround the receptor sites. It is impossible to directly monitor receptor-site drug concentrations in vivo, but plasma *total drug* concentrations reflect the equilibrium which exists between tissue, extracellular fluid, and plasma water drug concentrations, and plasma *free drug* levels reflect levels in the extracellular space.

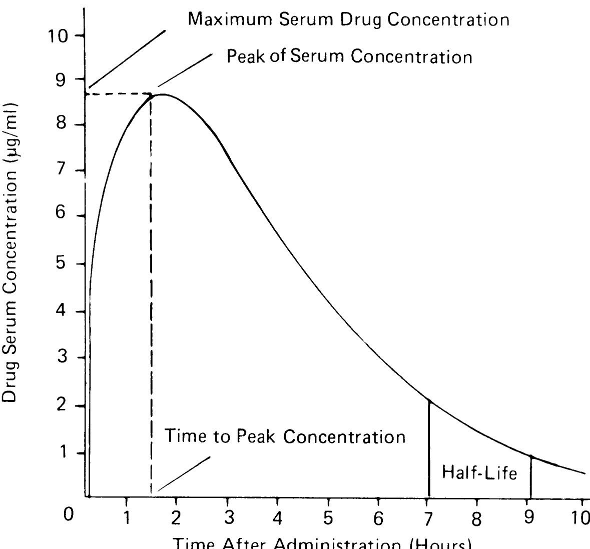


Figure 2 Theoretical dose response curve. Reproduced from the Syva Monitor with permission.

The *mechanism of action* of a drug refers to the actual biochemical or physical process which initiates a biological response at a specific site. The mechanism of action of most drugs depends upon their chemical interaction with a functionally viable component of some physiological system. However, since the exact molecular mechanism of action for most drugs remains obscure, theoretical models have been developed to explain this mechanism of action. The fundamental concept upon which these models are based is that drugs bind with intracellular macromolecular receptors by means of ionic bonds, hydrogen bonds, and van der Waals forces. Such a reversible combination is thought to form a drug-receptor complex of sufficient stability to alter the physiological response of the target system, consequently producing the observed pharmacological effect.

III. PHARMACOKINETICS

Anyone utilizing routing TDM must constantly keep in mind that the plasma concentration achieved and maintained following the administration of a fixed drug dosage is a direct consequence of the interactions of a wide variety of processes (Fig. 1). These include drug absorption, distribution, metabolism, and excretion, and the physiological status of the patient. All these factors are interrelated, each playing a role in determining the steady-state drug concentration which will be achieved on a fixed dosage regimen. The study of these interrelationships forms the basis of pharmacokinetics [9,10].

Pharmacokinetics is the study of the time course of drug and metabolite levels in different fluids, tissues, and excreta of the body, and of the mathematical relationships which can be utilized to develop models for interpretation of the blood concentration patterns observed in a given patient. In the practical sense, pharmacokinetics as a discipline represents an attempt to utilize mathematical models to predict the distribution and excretion patterns of drugs, usually at steady-state concentrations, in response to a given dosage regimen. Applied clinical pharmacokinetics can be used in treating patients receiving a given drug. The theoretical limitations of the models must be recognized however. For example, many models do not take into account multiple-drug therapy or the clinical status of the patient. Interactions between drugs can alter the kinetics of each and affect plasma drug concentrations as well. Therefore, unless specific clinical data from a given patient are available, these models should serve only as a general guideline.

Availability of drug monitoring techniques in biological fluids resulted in attempts to correlate a given mg/kg dosage of drug with the observed plasma concentration and clinical response in large patient populations. The fundamental assumption of these studies was that the patient was at steady state, that is, the intake of a drug was con-

stant over a period of time, and drug elimination, as reflected in the rates of drug metablism and excretion, was constant. Based upon data derived from these studies, a number of computer programs were developed that, given plasma concentration data with respct to time, would calculate the drug dosage necessary to achieve a given plasma drug concentration in a specific patient. Unfortunately, these programs and the information derived from them are not yet widely available to clinical chemistry laboratories or practicing clinicians.

The clinical application of these programs and information does not require a detailed knowledge of pharmacokinetics. However, an awareness of the terminology and fundamental principles is essential. The following descriptions and discussion of common pharmaceutical terms are designed to alert those engaged in TDM to the terminology of pharmacokinetics.

A. First-Order Kinetics

A process associated with drug utilization (clearance) exhibits first-order kinetics when there is a linear relationship between plasma drug concentration and total daily dose (mg/kg). Figure 3A graphically depicts that an increase in drug dose would be expected to result in a proportionate increase in plasma drug concentration.

B. Zero-Order Kinetics

When the rate of a process is independent of concentration, it is said to follow zero-order kinetics. The significance of zero-order kinetics becomes clearly apparent when a point is reached at which enzyme or transport concentration versus total daily dose (mg/kg) initially yields an apparently straight line, indicative of first-order kinetics, a sharp upward curve is seen as the saturation point is reached. The changes in the rates of drug clearance which occur beyond the saturation point, as represented by the disproportionate increase in plasma drug concentration following a given dosage increment, is the hallmark of zero-order kinetics (Fig. 3B).

Fortunately, in clinical practice, only a few drugs exhibit zero-order kinetics. For most drugs, the plasma concentrations achieved at therapeutic dosages are low relative to the concentration necessary to saturate the particular system involved. Therefore, first-order kinetics are observed throughout the therapeutic range. There are notable exceptions to this rule, however, since both phenytoin and aspirin exhibit saturation kinetics near the upper limits of the therapeutic range. For any drug that exhibits zero-order kinetics, a very small dosage increment may result in a clinically significant elevation of plasma concentrations. It is to be noted that even though the initial dose-response curve may appear linear in drugs with zero-order kinetics, the drug clearance is altered throughout the dosage range

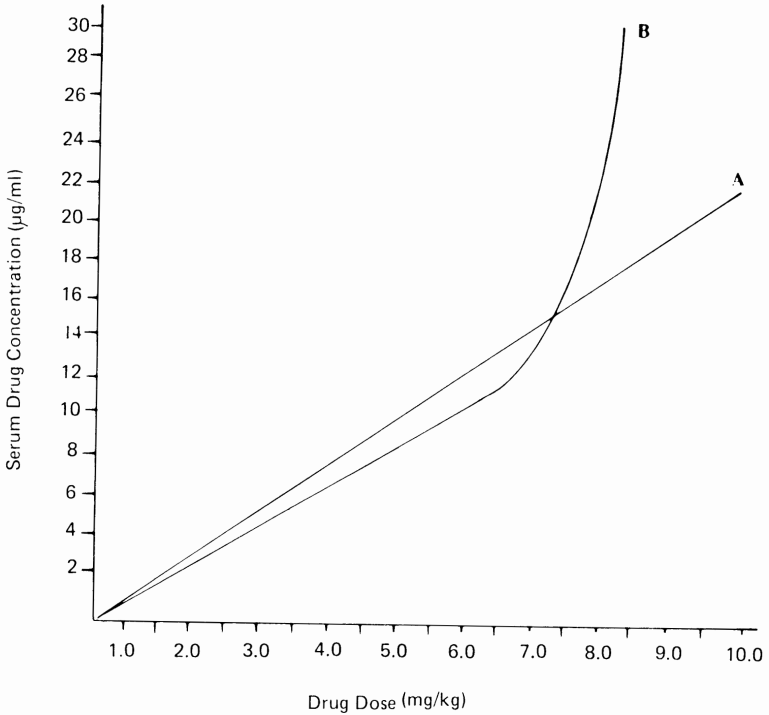


Figure 3 Relationship between drug dose and plasma concentration. (A) Dose-response curve for drug observing first-order kinetics (linear). (B) Dose-response curve for drug observing zero-order kinetics (nonlinear or saturation).

and at all plasma concentrations and does not parallel the kinetics observed in a first-order relationship.

C. Drug Half-Life

Drug half-life is also referred to as the elimination half-time, $t_{1/2}$. It is the time required for elimination of half the concentration of a drug present in the system, provided that no additional drug is administered following a given point in time. For example, if the concentration of phenytoin ($t_{1/2}$ = 24 hr) were 20 μg/ml, the time required to clear the drug to a concentration of 10 μg/ml would be 24 hr, provided no additional doses of the drug had been given. It must be emphasized that drug half-life is, in reality, a reflection of the individual rates of the several different processes which regulate drug clearance. The rates of drug metabolism and excretion are the primary determinants of the drug half-life in any given patient.

D. Fate of a Single Drug Dose

Following the administration of a single drug dose, a peak plasma concentration is reached when the absorption phase is almost complete. The plasma concentration then begins to decline, even as the drug continues to be absorbed. The rate of this decline in plasma concentration is dependent upon the rates of absorption, metabolism, and excretion of the drug. Once the absorption phase is complete, the rate of decline in plasma concentration is a reflection of the clearance (elimination) rate, which is the sum of the rates of excretion and metabolism of the drug. Following completion of the absorption phase, the half-life can be determined by measuring the decline in plasma concentration over fixed time intervals.

E. Steady States

When long-term oral therapy is initiated, the drug will continue to accumulate within the body until such time as the rate of clearance (which comprises all tissue distribution and the metabolic and excretion processes involved in drug disposition) equals the rate of administration. When the equilibrium between drug clearance and intake is achieved, the system is said to be at a *steady state*, that is, the amoung of drug ingested over a 24-hr period is equal to the amount of drug eliminated in the same 24-hr period. Over a period of time, body and plasma drug concentrations will increase exponentially until they reach a steady state or plateau. The time required to reach a steady state following institution of drug therapy is presented in Table 1. It requires seven half-lives of drug administration before a true steady-state concentration is achieved and stabilized. Steady-

Table 1 Percentage of Steady-State Drug Concentration Achieved at Each Half-Life Interval.

Number of half-lives	Percent of steady-state concentration
1	50
2	75
3	88
4	94
5	97
6	98
7	99

state processes are, however, 97% complete within 5 half-lives. As a practical rule, 5-6 times the half-life of any drug is the time required to achieve a steady state. For example, phenytoin, which has a half-life of 24 hr, requires a period of 24 × 5.5 or 132 hr (5-6 days) to achieve a steady state. In contrast, drugs such as primidone or valproate, which have half-lives of 6-8 hr, require only 33-44 hr to reach a steady state.

It is important to note that the same principles which govern the gradual accumulation of a drug to a steady state also apply when drug therapy is discontinued. For instance, if plasma phenytoin is at a steady-state concentration, and drug administration is stopped, there will be a period of 5-6 days, or 5-6 half-lives, before the drug is completely eliminated from the body. This is why drugs with prolonged half-lives can still be detected in plasma for 3-4 weeks after administration of the last dose. For example, phenobarbital, with a half-life of 4 days, requires 22 days for complete elimination. Bromides, with a half-live of 12 days, require 66 days for complete elimination (or to reach steady state).

Plasma concentrations for drugs with first-order kinetics during the steady state are linearly related to dose. For any change of dosage, after a steady state has been achieved, the principles regulating the time required to achieve a new steady-state stage still apply. To illustrate this important principle, if the maintenance dose of a drug were doubled, the new steady-state concentration would not double until the completion of 5-6 half-lives. If a plasma drug concentration is determined before achievement of a steady state, for example, after 2 half-lives, it will not reflect the true steady-state concentration of the drug.

It is essential to understand that the full therapeutic effect from a given dose is not achieved until steady-state concentrations are reached. Therefore, before a given dosage regimen is considered a failure, the clinician should be sure that steady-state concentrations have been achieved.

F. Factors Which Alter Individual Drug Disposition Patterns

1. Patient Noncompliance

It has been suggested that over 60% of all patients do not take their medications in the manner prescribed by their physicians. The most common cause of suboptimal drug concentrations, and consequent failure to achieve the desired therapeutic response, is patient noncompliance. Whenever a patient presents with consistently low plasma drug concentrations, noncompliance should be considered the probable cause. Noncompliance can usually be demonstrated by careful supervision of the patient's daily drug intake over a specified time interval

(usually 5 half-lives of the drug) with routine monitoring of serum drug concentrations at appropriate intervals. If there is a progressive increase in serum drug concentration over the time interval selected, the patient has been noncompliant.

The administration of the recommended or average total daily dose of a given drug without taking into account the numerous factors which alter drug disposition in each patient can also lead to consistently low serum drug concentrations. Failure to individualize drug therapy (physician noncompliance) is often responsible for suboptimal drug concentrations. If the serum concentrations remain low under supervised intake, other causes such as drug malabsorption or rapid drug metabolism should be suspected.

2. *Drug Absorption*

Some patients receiving appropriate drug doses will have consistently low plasma drug concentrations. Generally. these patients are classified as either noncompliant or as fast drug metabolizers. However, before classifying someone as a fast metabolizer (see below), the patient's ability to absorb the administered drug should be evaluated. The entrance of drugs into the general circulation following either i.v. or i.m. administration is generally rapid and circumvents the problems associated with drug absorption following oral administration; however, most drugs are administered orally. Following oral drug administration, the type of drug preparation, drug solubility, concomitant administration or other drugs, whether or not the drug is taken with meals, and the presence of diarrhea or constipation can all alter the amount of drug which will be absorbed from the gastrointestinal tract following a single dose.

Malabsorption of an orally administered drug can often be confirmed by measuring serial plasma drug concentrations at given time intervals after parenteral administration of the prescribed dose. If altered absorption is present, the maximum plasma concentrations and observed drug half-life following the i.v. dose will be significantly higher than those achieved following the same dose administered orally. Conversely, if the patient's problem is fast drug metabolism, there will be no significant differences in the plasma concentrations achieved or the observed half-life regardless of the route of administration.

3. *Drug-Plasma Protein Binding*

Upon entering the systemic circulation, any protein-bound drug will bind to plasma proteins, and an equilibrium between free and bound drug will be established. By definition, the *bound drug* is that portion of a drug bound to plasma proteins. Bound drug in unable to cross cell membranes and consequently exerts no biological effect. The unbound or *free* drug is dissolved in the plasma water and can be transported across cell membranes. Only the free drug can cross bio-

logical membranes and interact with specific receptors to elicit a biological response. Each drug has its own characteristic protein-binding pattern which is dependent on its physical and chemical properties. As a general rule, acidic drugs are bound primarily to albumin, and basic drugs to globulins, particularly α_1-acid glycoprotein.

A drug may be either tightly or loosely bound, as determined by its affinity for plasma proteins. A weakly bound drug can be displaced from its protein sites by binding one with greater affinity for the plasma protein-binding site. Protein binding of a drug is also dependent on the physical characteristics of the plasma proteins and on the presence or absence of fatty acids or other drugs in the blood. Tightly bound drugs will not be displaced, but a weakly bound drug can be displaced quite rapidly from its protein-binding sties by elevated free fatty acids or by another drug. It is important to recognize that even though the total serum drug concentration may remain unchanged, displacement of a drug from its plasma protein-binding site can elevate free-drug concentrations and result in clinical toxicity.

Certain disease states can significantly alter drug protein binding. For example, uremic patients lack the ability to completely bind drugs to plasma proteins. In the case of phenytoin, these patients range from those who can bind no phenytoin to those who can bind only 60-70% of the phenytoin present in plasma. Clinically, this means that in a patient who lacks the capacity to bind phenytoin (i.e., whose phenytoin is 100% free), concentrations of 1-2 μg/ml would result in clinical effects equivalent to 10-20 μg/ml in persons whose phenytoin is normally bound (i.e., 10% free), and plasma concentrations above 2.5-3.0 μg/ml would result in phenytoin toxicity. Altered drug-binding requires careful monitoring of all drugs administered in patients with abnormal renal function.

In patients who present with wither clinical toxicity or a nontherapeutic response, when total plasma concentrations are known to be optimal, altered protein binding should be considered. Until recently, determination of protein binding was a time-consuming and tedious procedure. Since only the free drug crosses into the saliva, the protein-binding status of a patient can be assessed indirectly by measuring salivary drug concentrations. Nevertheless, caution is indicated. Salivary levels are a good indicator of free drug levels for any drug that has an ionization constant (pK_a) significantly different from the pH of plasma, for example, phenytoin. However, for drugs with pK_as similar to plasma pH (e.g., phenobarbital), salivary concentrations will not reflect the true free-drug concentrations. In addition salivary drug levels will not reflect the actual free concentration of drugs which are actively transported into the saliva. The recent development of rapid ultrafiltration systems which directly assess free plasma drug concentrations should enhance our ability to identify patients who lack the ability to bind drugs to plasma proteins normally.

4. Drug Metabolism

Any foreign compound that enters the body must be eliminated. Drug elimination mechanisms become more complex as one proceeds up the phylogenetic scale from fish to man. There is a progressive increase in the ability of the body to alter foreign compounds into compounds which are more water-soluble (less fat-soluble) and thus more readily excreted. It is generally believed that the ability of the liver to metabolize drugs evolved as a mechanism for detoxifying poisonous substances ingested with food.

The drug-metabolizing enzymes of the liver are nonspecific and interact with a wide variety of chemical structures. Metabolites of many drugs are conjugated within the liver to either glucuronic acid, amino acids, or sulfates, thus increasing water solubility even more, and consequently, the rate of renal excretion. For example, *p*-hydroxyphenytoin, the major metabolite of phenytoin, is conjugated with glucuronic acid. This conjugation increases its water solubility almost 100 times.

Most drug metabolism takes place within the microsomal fraction of the hepatocyte. The microsomal enzyme systems are also responsible for the metabolism of endogeneous steriods. These systems are not designed to recognize specific drugs; rather, they act upon classes of compounds with similar structures. The same enzyme that is responsible for the hydroxylation of phenytoin is also responsible for the hydroxylation of many other drugs containing an appropriate phenyl ring. Therefore, when phenytoin is administered simultaneously with one of these drugs, there may be some clinically significant alterations of drug concentrations that are a direct consequence of competition for metabolic sites. Clinically, one would expect to see higher serum concentrations of the drug with the least affinity for the enzyme. Phenytoin has a very low affinity for microsomal enzymes. Thus, administration of a drug with a greater affinity for the enzyme than phenytoin will decrease phenytoin's rate of metabolism, and plasma phenytoin concentrations will become elevated. One should realize, however, that not all compounds are metabolized at the same site. One can be a fast metabolizer of one group of compounds and yet normally metabolize others. Similarly, one compound may displace phenytoin from its metabolic site whereas another will have no effect.

One characteristic of the hepatic microsomal system is that it can be induced to metabolize drugs at a faster rate. As increasing doses of drug are administered, the body, in its attempt to eliminate the drug, synthesizes new proteins, in the form of enzymes capable of metabolizing that agent. Increased activity of drug-metabolizing enzymatic systems is not necessarily induced with every dosage increment or with the addition of another drug to the patient's regimen. There is a maximum rate at which protein synthesis can occur. Thus, if a patient has been regularly receiving a drug with known enzyme-induc-

tion properties, it does not follow that a second drug of similar structure added to the patient's therapeutic regimen will cause a marked increase in the rate of metabolism of both the first and second drug.

Genetic factors play a major role in determining the ability of a patient to metabolize drugs. Individuals of different ethnic origins as well as individuals in certain families metabolize drugs (e.g., phenytoin or isoniazid) at a faster or slower rate than the general population. A fast drug metabolizer will require a greater daily dose (mg/kg) than will a "normal" individual to achieve the same serum concentration necessary for eliciting the desired therapeutic response. A slow drug metabolizer given standard drug dosages will invariably exhibit drug toxicity.

Absolute identificaiton of fast and slow drug metabolizers depends upon the quantitative identification of urinary drug-metabolite excretion profiles as well as on the serial determination of plasma drug concentrations. Generally, plasma drug concentrations of slow metabolizers will be significantly higher than would be observed in the general population receiving the same mg/kg/day dosage. Consistently high plasma concentrations in patients on normal or low drug doses is suggestive of slow drug metabolism. However, a drug interaction or disease process that blocks drug metabolism will also result in elevated plasma concentrations. On the other hand, fast drug metabolizers usually exhibit consistently low plasma concentrations on standard dosage regimens. Since plasma drug levels in noncompliant patients mimic those observed in fast metabolizers, there is a tendency to identify noncompliant patients as fast metabolizers. Use of plasma drug concentrations alone to identify fast and slow metabolizers can be misleading. Urinary excretion patterns help to clarify whether a given patient's findings are due to metabolic alterations, noncompliance, or another problem.

Generally, drugs are metabolized from a pharmacologically active agent to an inactive product, incapable of eliciting a given therapeutic response. There are exceptions to this rule: Some organic compounds, when metabolized, have a greater biological activity than the parent compound. For example, diazepam is rapidly metabolized to desmethyldiazepam, which is the most active antianxiety agent of all the diazepam metabolites. As a general rule, when a compound has a less polar active metabolite, the half-life of the active metabolite is significantly longer than that of the parent compound. Such is the case with procainamide and *N*-acetyl procainamide (NAPA). The half-life of procainamide is 3-4 hr, whereas NAPA has a half-life of 6-9 hr in patients with normal creatinine clearance. This means that there will be an accumulation of NAPA, the active metabolite, within the system and at its site of action.

The clinical status of a patient can also dramatically alter drug utilization patterns. Hepatitis can impair the metabolism of drugs. If the liver has lost its reserve capacity, patients with hepatitis can be-

come severely intoxicated when given drugs dependent upon hepatic degradation. Congestive heart failure can significantly alter the distribution of drugs to tissues, thus precipating altered drug utilization and response patterns.

5. Renal Excretion

Urinary excretion is the major pathway for the elimination of drugs and their metabolites. For any drug which is not extensively metabolized, changes in renal function will alter that drug's plasma concentrations. If renal function is impaired, drug plasma concentrations can become elevated.

Uremic patients and those with congestive heart failure have decreased renal drug clearance. Interestingly, drug metabolites are so water-soluble that a significant decrease in urinary output will not result in increased plasma concentrations of most conjugated drug metabolites.

IV. GUIDELINES FOR ROUTINE THERAPEUTIC DRUG MONITORING

The ultimate responsibility of the laboratory engaged in routine TDM is to assure that all information relevant to the patient's pharmacological profile is available for utilization by the clinician to individualize a given patient's therapeutic regimen [1-10].

Clinical pharmacokinetics is a valuable tool for understanding and interpreting the response of an individual patient to a given drug regimen. A number of texts containing detailed mathematical derivations of the fundamental principles of clinical pharmacokinetics are available. Computer programs which apply these principles to dosage calculations for individual patients have been developed. These programs can be utilized for any given drug to calculate the expected plasma concentration which will be achieved over a fixed time interval following a given dose, provided the patient's plasma concentration at a given time interval after the last drug dose is available. These programs and the information derived from them, unfortunately, are not widely available to the clinical laboratory or to the practicing clinician, but a series of simple guidelines exist which will generate approximately the same information as the computer programs without the necessity of complex mathematical formulas or computer programs. The following sections deal with (a) information which should be available in order to make decisions regarding optimal drug administration, and (b) simple procedures for interpreting and applying the laboratory results obtained with respect to a given drug.

A. Patient Information Necessary for Interpreting Drug Concentrations

Wide individual variability exists in patient utilization of drugs as a direct consequence of genetic factors, multiple drug therapy, age, and weight.

In order to derive as much information as possible about the pharmacological status of the patient, each laboratory engaged in TDM should have the following information available at the time any drug is monitored.

1. Patient's age. It is clearly established that there are marked age-dependent differences in drug utilization; in particular, the transition ages between neonate and infant, child and adolescent, and adult and geriatric must be considered.

2. Patient's weight. The weight of the patient is essential for mathematical calculations of the relationships between the drug dose, plasma concentration, and drug clearance.

3. All drugs which the patient is receiving. Knowledge of the drugs which the patient is receiving, in addition to the agent being monitored, is essential for identification of potential drug interactions which might alter plasma concentrations as well as for the identification of compounds which may interfere with a given analytical technique.

4. Total daily dosage of drugs. A knowledge of the total daily dosage for each drug administered is necessary to mathematically determine the patient's total daily drug dose in milligrams per kilogram. Without this information it is impossible to correlate the patient's actual plasma concentration with his expected plasma concentration. Knowledge of the mg/kg dose allows a prediction of the patient's expected plasma drug concentration to be made by calculating with the concentration:dose ratio (CDR). Predicted drug concentrations can then be correlated with the observed (measured) drug concentration to provide an indication of the patient's pharmacological status.

5. Critical time intervals. The time at which the last dose of drug was administered and the time at which the blood specimen was drawn are essential elements of data. Without this information, it is difficult to assess whether the actual plasma concentration represents a peak or trough level. A knowledge of the actual sampling time and dosage interval is extremely important for accurate interpretation of plasma concentrations of drugs with short half-lives, such as theophylline and lidocaine.

6. Clinical status of the patient. It is well established that acute or chronic disease can dramatically alter drug utilization patterns. Awareness of the patient's current clinical status is particularly important for regulation of drug therapy in patients with hepatitis or renal failure. Without the knowledge of the clinical status of the patient, it is impossible for those interpreting drug concentrations to distinguish an altered drug utilization pattern which is associated with a given

disease state from other factors (noncompliance, drug interactions, etc.) which can be present with a similar pattern.

B. Time of Drawing TDM Specimens

A question which often arises is the time of drawing the specimen. As a rule, specimens for TDM should be drawn at a trough, that is, when the concentration should be the lowest for that dosing interval. Measurement of peak concentrations following oral administration is difficult because of the marked individual variability in drug absorption patterns. Peak levels are indicated, however, in certain situations following i.v. drug administration and have been reported to be of value in the monitoring of antibiotics, theophylline, and certain antiarrhythmic drugs.

Although population studies provide useful guidelines, successful TDM is based on individualization of drug therapy which is accomplished by assessing the significance of the plasma concentration, the clinical status of the patient, and the therapeutic goals.

Selection of the time a specimen is drawn in relation to drug administration should be based on the pharmacokinetic properties of the drug and dosage form. Patient should be at or near steady state when sample is drawn. This is achieved when the drug has been administered at a constant rate for 5-6 half-lives. After dose adjustment, time should allow for equilibrium to be reestablished with the new dosage regimen before another specimen is drawn. Specimens drawn immediately before administration of the next oral dose provide through serum levels for drugs administered on a chronic basis; the trough level should ideally be above the minimum effective serum level. Specimens for peak levels are generally drawn 15-30 min after i.v. administration, 1-2 hr after i.m. administration, and 1.5 hr after oral administration (depends on rate of drug distribution). When the specimen is going to be drawn during an infusion, the sample should be taken from the opposite limb.

C. Interpretation of Plasma Concentration

Anyone involved in the utilization of information derived from TDM must always bear in mind that the interpretation of plasma drug concentrations must always be carried out in conjunction with an assessment of the clinical status of the patient! Rather than therapeutic ranges, the clinician should be concerned with optimal concentrations. The optimal concentration (therapeutic range) of a drug is defined as that concentration of drug present in plasma or some other biological fluid or tissue which provides the desired therapeutic response in most patients. It is to be emphasized that the severity of the disease pro-

cess determines the amount of drug necessary to achieve a given therapeutic effect. Thus it is quite possible that a given patient may achieve the desired therapeutic effect at a plasma concentration well below the optimal range. Others may require levels above those usually considered optimal to achieve the desired clinical effect and may tolerate these levels without evidence for toxicity. Still others will not achieve the desired therapeutic effect even when plasma concentrations are elevated into the toxic range. If the desired therapeutic effect is achieved at usually suboptimal plasma concentrations, there is no reason to increase the dose of medication in that patient. In a second patient who does not achieve the desired effect at a suboptimal plasma level, the dose of that drug should be increased; every attempt should be made to avoid the prescription of additional drugs simply to increase the plasma concentration into what is commonly referred to as "the therapeutic range." Obviously, the interpretation of plasma drug concentration must take into account the various factors which can alter the steady-state plasma concentration achieved on a given dosage regimen.

1. *Indications for Monitoring Plasma Drug Levels*

Plasma drug levels should be monitored for the following reasons:

When a drug has a narrow, well-defined therapeutic range.
When noncompliance is suspected.
When the desired therapeutic effect is not achieved or when symptoms of toxicity are observed.
When there are large interindividual variations in drug utilization or metabolism.
When drug utilization is altered as a consequence of secondary disease or physiological state.
When drug interaction is suspected.
When there is a need for medicolegal verification of treatment.

2. *General Factors Influencing Interpretation of Assay Data for TDM*

Patient compliance, including dosage error and wrong medication
Absorption via route of administration
Drug distribution
Biotransformation
Excretion
Genetic variability
Pathophysiological factors (acute or chronic disease)
Drug interactions
Drug tolerance
Inappropriate drug effects

3. *Information Needed for Interpreting Drug Levels*

Patient age, weight, and sex
List of all of the drugs which the patient is receiving
Total daily dose of all drugs
Dosage regimen and dosage form of each drug
Time the last dose of drug (the level of which is being requested) was administered
Time the specimen was drawn
Clinical status of the patient

D. Determining Dosage Intervals

A steady-state drug concentration is usually maintained in an individual patient by various combinations of total drug dosage and dosage-intervals. Generally, in order to maintain a smooth, constant, steady-state drug concentration without excessive fluctuations, dosage intervals should be half of each particular drug's half-life. To illustrate this in practical terms, phenytoin would be given every 12 hr since its half-life is 24 hr, whereas primidone would be given every 3 hr, since its half-life is 6-8 hr with polytherapy, or every 8 hr, since its half-life is 15 hr with monotherapy.

Short dosage intervals (3-4 hr) are often impractical in outpatients, but drugs with short half-lives should be administered at least once each half-life. The principal idea is to maintain the valley (or trough) drug concentration within the therapeutic range, that is, above the minimum effective concentration (MEC), without the peak concentration reaching toxic levels. As long as the dosage schedule and the interval between doses are selected so that there are no significant fluctuations between peak and valley concentrations of drug during the dosage interval, an appropriate steady-state concentration will be maintained. If the dosage interval is too long relative to the half-life of the drug, the plasma concentration just prior to the next dose may be insufficient to provide the desired therapeutic effect. To apply this concept to epilepsy, for example, a patient whose phenytoin level falls from 14 μg/ml to 9 μg/ml may have a seizure at the lower level but not at the higher. For any drug, it is possible to maintain a plasma concentration within the optimal therapeutic range at all times by adhering to appropriate dosage schedules.

If a patient's drug absorption is very rapid, or if dosage intervals are excessively short, he or she may experience periods of drug intoxication which may present clinically as the side effects normally associated with that drug. These symptoms usually appear transiently at fixed intervals following drug administration throughout the day. The toxicity is attributed to a peak plasma concentration above the optimal range shortly after drug administration. Such side effects

can often be eliminated by extending the dosage interval to assure that peak concentrations are not excessive.

E. Monitoring Steady-State Drug Concentrations

When long-term oral therapy is initiated, the drug will continue to accumulate within the body until such time as the rate of drug clearance (elimination) is in equilibrium with the total daily drug intake. Drug clearance encompasses all absorption, distribution, and metabolic and renal processes involved in drug disposition. Over a period of time, body and plasma drug concentrations will increase exponentially until they reach a steady state or plateau.

We again emphasize that the time required to reach a stabilized steady state is 7 half-lives following institution of drug therapy; steady-state processes are, however, 97% complete within 5 half-lives. If the prescribed drug dosage is changed after a steady state has been achieved, the principles regulating the time required to achieve a new steady-state plateau still apply (5X the drug half-life). For instance. if the maintenance dose of a drug were doubled, the new steady-state drug concentration would not be doubled until the completion of 5-6 half-lives. Only at steady state is the full therapeutic effect of that dose achieved.

If a plasma drug concentration is determined before achievement of a steady state, for example, after only 2 half-lives, it will not reflect the true steady-state concentration of the drug. Measurement of plasma concentrations before a steady state is achieved does not yield as much clinically useful information with respect to the patient as do levels measured at steady state.

It is possible, if one knows the time of initiation of drug therapy, to extrapolate predicted steady-state plasma concentrations by correcting for the number of half-lives expired before sampling. This technique, however, provides only a rough estimate of the expected steady-state concentrations.

F. Consideration of Half-Life Variables

Drug half-life, by definition, is the time required for elimination of half the plasma concentration of drug present at an initial starting time. If must be remembered that half-life is, in reality, the elimination half-time of a drug and reflects the various factors which determine that drug's clearance. Since half-life is dependent upon the rates of drug metabolism and excretion, it is the sum total of these two processes which define a drug's clearance (elimination) rate. Clearance rate does not necessarily refer to the actual elimination of the drug from the body since drug metabolism can convert drugs from pharmacologically active to pharmacologically inactive compounds. Thus the pharmacological activity of the drug may be eliminated, even though the drug's metabo-

lite is still present in the body. It is to be emphasized that most analytical techniques for routine TDM today measure the parent compound and do not measure drug metabolites. Nevertheless, a sudden change in measured steady-state drug concentrations usually serves as an indicator of altered drug disposition of the pharmacologically active (parent) compound.

For example, the addition of sodium valproate to the regimen of a patient who is receiving phenytoin may result in a marked decrease in total phenytoin concentrations. This decrease is a direct consequence of the displacement of phenytoin from its plasma protein-binding sites. The displaced phenytoin is rapidly converted to its inactive metabolite, *p*-hydroxyphenytoin. The observed fall in total phenytoin levels is indicative of an altered rate of phenytoin disposition.

G. Multiple-Drug Therapy Affects Drug Disposition

Most clinically significant drug interactions are readily identifiable in the presence of elevated plasma concentrations of a given drug. Generally it will be observed that the interfering drug has a metabolic pathway which is similar to that of the drug being monitored. In addition, multiple-drug therapy can also alter absorption, protein binding, and renal clearance of a given agent. Change in any of these factors can result in altered steady-state concentrations.

Any factor which alters drug half-life (clearance) will alter the drug's steady-state concentration. During multiple-drug therapy, two drugs may compete for the same metabolic site. This competition will decrease the rate of metabolism of the drug which is excluded from the site and prolong its half-life. Since the half-life is prolonged (i.e., clearance is decreased), a new, higher steady-state drug concentration will be achieved and maintained, as long as the multiple drug therapy is continued.

H. Disease States Alter Drug Disposition

Drug clearance is dramatically altered during renal and liver disease because the elimination rates of the drugs are changed. Consequently, new steady-state levels will be achieved which may differ significantly from those observed in healthy individuals. One must always consider the clinical status of the patient when interpreting plasma drug concentrations.

I. Drug Disposition Is Altered in Healthy Individuals Due to Pharmacogenetics

As noted above, drug clearance is significantly regulated by genetic factors. In a large population of patients one could predict that if the entire population were given the same mg/kg dosage of a drug,

there would be marked differences in the ability of individuals within the population to utilize the drug. These genetic differences will be reflected in a marked variability of the steady-state plasma concentrations observed in this population.

For example, in a population of patients receiving phenytoin at a standard therapeutic dose of 5 mg/kg/day, one would theoretically expect all patients to have a therapeutic drug level of 15 μg/ml. In reality, plasma concentrations will range from 0 μg/ml, which suggests drug malabsorption, patient noncompliance, or fast drug metabolism, to levels of 40-50 μg/ml, which may indicate drug reactions, hepatic or renal disease, genetically slow drug metabolism.

As an example, consider the incidence of fast and slow metabolism in patients receiving isoniazid, a drug commonly used in the treatment of tuberculosis. Approximately 40% of all Caucasians are rapid acetylators of isoniazid. In contrast, over 90% of Japanese and Eskimos are rapid acetylators. This genetic variability requires individualization of therapeutic regimens to assure the maintenance of optimal isoniazid concentrations in the different populations and individuals within the population.

J. Age Alters Drug Disposition

The many factors involved in determining the steady-state plasma concentrations of a drug after a fixed dosage regimen have been reviewed above. Detailed information related to this subject is available in any medical pharmacology text. Ultimately, the pharmacological (or biological) effect observed clinically after a given drug dosage is determined by the concentration of drug at the receptor site. This concentration can be changed by altering the rate of drug absorption, the degree of protein binding, the rate of drug metabolism, or the rate of renal excretion of the drug. Such changes can occur as a consequence of normal physiological development, an alteration in physiological systems as a consequence of disease, or an alteration due to administration of a pharmacologically active agent.

As we have noted earlier, essentially from birth until death the efficiency of the physiological systems constantly decreases in any given individual. This continual decrease occurs rapidly during some stages of growth and development, and slowly during others. Age-related differencces in drug disposition are a reflection of changes in normal physiological function. It is to be emphasized that although the rate of drug disposition in children is increased, the optimal drug concentration of most therapeutic agents necessary to produce the desired therapeutic response is similar to that observed in adults. Therefore, because of the faster drug clearance in children, it is necessary to prescribe larger drug doses (in mg/kg) to the pediatric population in order to achieve and maintain optimal drug concentrations, whereas in

geriatric patients lower drug doses are usually necessary to avoid drug toxicity.

V. CONCLUSION

The clinical utility of TDM as an adjunct to the management of patients is firmly established. The number of drugs routinely monitored will continue to grow, and the success of this expansion will depend on the application and development of current technologies as well as the development of new ones. Over the next few years the major technologies for drug monitoring will likely be the homogeneous enzyme immunoassay system and HPLC because of their reliability and ease of operation. The continuing education of both physicians and laboratory personnel with respect to the new technologies and their clinical applications is essential to ensure the continued expansion of TDM and its ultimate application to improving patient welfare.

Author's Note

The material presented in this article represents a compilation from reviews by the author over the past several years. Extensive portions of these reviews are included. The references listed below represent articles and books covering all aspects of TDM.

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3

LIQUID CHROMATOGRAPHY AND OTHER METHODOLOGIES FOR THERAPEUTIC DRUG MONITORING AND TOXICOLOGY

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I. INTRODUCTION

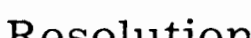
Drug analysis may be performed by a wide spectrum of techniques [1-16]. The purpose of this chapter is to establish a basic and current understanding of these techniques in order to facilitate the clinical laboratory's selection of the appropriate method for drug monitoring and toxicology. Through an understanding of current immunoassays, chromatographers can better define the roles of LC, GC, and thin layer chromatography. Thus this chapter first describes the two most popular chromatographic techniques: liquid chromatography (LC) and gas chromatography (GC). The section on LC includes a brief discussion of the principle in order to establish a basic understanding of LC processes. Instrumentation components will be reviewed with an emphasis on the more recent developments relevant to drug analysis. The second half is devoted exclusively to drug immunoassays. For more established and traditional assays such as radioimmunoassay and enzyme-multiplied immunoassay techniques (EMIT), and for more recently introduced techniques such as nephelometric inhibition immunoassay (NIIA), a brief description is provided. Readers are also referred to the authoritative and updated references. A more detailed discussion will be devoted to a newly introduced technique, fluorescence polarization immunoassay (FPIA), based upon the author's own experience. With such a review, it may be possible to better define the role of LC as a tool for clinical drug assays in relation to other available instrumentation.

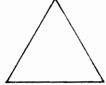
II. LIQUID CHROMATOGRAPHY

Since the advent of LC in its modern form from the late sixties to the early seventies, numerous books and articles had adequately treated the subject [9,17-21,25-27]. More recently, however, Kabra [10] and Koenigsberger [12a] discussed the principles of LC and other topics in relation to therapeutic drug monitoring. To avoid overlap with the works just cited, this section will be devoted to a brief but essential discussion of the principle of LC, with emphasis on therapeutic drug monitoring and toxicology.

A. Principles

In order to measure drugs and their metabolites (analytes), analytes must be separated from each other and from other endogenous compounds. Due to the differences of analyte interaction with packing and mobile phases, the analytes are separated in to various zones. For two structurally similar analytes, such as a parent drug and its metabolite [for example, maprotiline (MAP), a tetracyclic antidepressant, and its metabolite, N-desmethyl maprotiline (ND-MAP)], complete resolution may be achieved [29] (see Fig. 1). Such a chromatographic separation represents a compromise between three factors: resolution, capacity, and speed [17], as shown by the chromatographer's triangle:





Speed Capacity

The resolution between two such peaks is estimated by the following equation:

$$R = \frac{2(t_{R2} - t_{R1})}{W_2 + W_1} \tag{1}$$

where t_{R1}, t_{R2} = retention times of the retained components (e.g., MAP and ND-MAP), and W_2 and W_1 = widths in the units of time measured at the base.

Resolution may be achieved by adjusting the following three factors, as shown by Eq. (2): efficiency (A), selectivity (B), and capacity (C).

$$R = \underbrace{\frac{1}{4}\sqrt{N}}_{A}\;\underbrace{\left(\frac{\alpha - 1}{\alpha}\right)}_{B}\;\underbrace{\left(\frac{k'}{k' + 1}\right)}_{C} \tag{2}$$

Figure 1 (Top) N-desmethylation of MAP. (Bottom) Chromatograms of plasma extracts: (A) without drug; (B) with MAP (2) and ND-MAP (1) standards; (C) from a patient receiving 200 mg of MAP daily (3 = internal standard). (Reprinted with permission from *Clinical Chemistry*, Ref. 29.)

Column efficiency can be expressed by the number of theoretical plates, N, where

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{W}\right)^2 \tag{3}$$

where

σ = peak standard deviation and

W = peak width at the baseline.

In order to compare the efficiency at various column lengths, the height equivalent to the theoretical plate, HETP or H, may be defined as follows:

$$H = \frac{L}{N} \tag{4}$$

where

L is the length of the column in meters.

For an efficient separation, a small H is desired, usually 0.01-1.00 mm. For a 10-μm particle column, a flow-rate of about 2 ml/min would result in the most efficient separation with minimum H. For a 5-μm column, the optimum flow-rate is about 1.5 ml/min.

For a kinetic interpretation, column efficiency is governed by zone spreading, attributed primarily to the following three sources: (1) multiple paths, (2) molecular diffusion, and (3) mass transfer, as shown by Eq. (5).

$$H = A + \frac{B}{U} + C_s + C_m \tag{5}$$

Multiple path refers to the various flow-velocities through a column. By following the various flow streams, some molecules move into slower streams and stay behind. Thus these flow velocities result in zone dispersion of the analytes. This contribution is designated as A.

Molecular diffusion of the anaytes in the mobile phase depends on the time, the tortuosity factor of the packing, and the diffusivity of the mobile phase. The contribution to the plate height is designated as B/U, where U is the flow-velocity.

Mass transfer refers to the analytes passing into and out of the packing. Due to various transfer rates of analytes, some molecules migrate ahead of the others. This phenomenon occurs in both the stationary and mobile phases, and their respective contributions are designated as C_s and C_m.

Other contributing factors to H are extra-column band broadening, a result of the length and internal diameter of the interconnecting

tubings (they should be as small as possible); and sample size. Optimal values range from 20 μl to 100 μl for TDM-LC separation, based upon the author's experience and numerous published drug assays. (Note a 2-ml injection was used for "FAST-LC" analyses.)

Column selectivity, α, of the B term of the resolution equation (2) may be expressed as:

$$\alpha = \frac{t_{R2} - t_M}{t_{R1} - t_M} = \frac{K_2}{K_1} \tag{6}$$

where t_{R2}, t_{R1}, and t_M are retention times of components 2, 1, and mobile phase, and K_2 and K_1 are the distribution coefficients of component 2 and 1, respectively.

To optimize column selectivity, the following factors may be considered: mobile-phase strength, pH (2-8 for silica-based columns), temperature, packing, and special chemical effects. Mobile-phase strength for a typical reversed-phase drug assay depends primarily on the percentages of aqueous component and of the organic modifier (such as MeOH or ACN). The mobile phase pH is adjusted so that the analytes may exist in either free or ionized form. A special application by Vandemark [28] is the use of alkalinized pH mobile-phase, suppressing the tricyclic ionization. Tailing is minimized, and thus α is increased.

Elevated temperature may be effectively used in changing column selectivity. An increase in temperature results in the lowering of k' due to an increased rate of interaction between the analytes and the column packing. From the author's experience [29] and that of others [30], a temperature of 50-60°C may be used without noticeable detrimental effect on column life.

Another important factor is the functionality of column packing, such as C_8, C_{18}, NH_2, CN, and phenyl. The optimal use of some of these columns will be discussed later on in the section on column selection (II.B.c). Special chemical effects or secondary equilibria such as ion-pairing or complexation also dramatically alter selectivity [31].

The third major contribution to R is the capacity factor, C. The relationship of R to k'/1 + k' dictates that as k' exceeds 10, the relative increase in R is greatly minimized. For example, for k' = 10, the term k'/1 + k' = 0.91, and for k' = infinity, k'/1 + k' = 1.00. Thus it is desirable to achieve a separation so that $2 < k' < 10$. The analytes may then be adequately separated from the solvent front, the other endogeneous interferences, and from structurally similar metabolites. Moreover, the analysis would not be so long as to result in peak broadening, in addition to the disadvantage of lengthy analysis time. The factors contributing to k' for a given column packing would include the mobile-phase strength, temperature, and pH. By changing the three factors of Eq. (2), an optimal separation for TDM and toxicology may be achieved with k' = 2 to 10, in less than 10-15 min.

B. Instrumentation

The LC design since its inception has followed two approaches, modular and cabinet. The advantages of modular design are versatility in changing components and in attaching other accessories to the instrument, easy access for preventive maintenance or repair, space efficiency, and partial breakdown not affecting the whole system. But such systems suffer from minor problems of appearance, with tubing and connections readily showing, and from possible problems with interfacing, encountered during automation later on. With the cabinet design, the various components are placed in predesigned positions, but the cabinet is usually bulky and components may be hard to reach for servicing. The author's drug analysis laboratory has both kinds of instruments and has not been able to favor one over the other. A sensible choice may be made, not based on the design, but rather on the space allocation, the budget, the requirements for various assays, the experience of personnel, and the possibility of future expansion, automation, and computer interfacing.

If the drug assay laboratory performs a few (less than ten) easy assays such as theophylline, and the work load ranges from low to moderate, a simple isocratic LC with a fixed-wavelength UV detector may suffice. However, for an advanced drug assay laboratory with high work loads and large variety of drug assays, a more sophisticated, automated LC with computer interfacing is desirable. The monitor should control the operation of the pumps for flow programming or gradient elution, temperature, injection, and possible wavelength selection at various time intervals for ratioing purposes. In order to avoid interfacing problems, the monitor should be manufactured by the same company which supplies the other LC components. Recently, microcomputers have been modified to offer control/monitor capability. Considering the low cost of such computers relative to the cost of some HPLC and personnel, it may be worthwhile to consider such a purchase. For further discussion on this subject, the reader is referred to Scott's chapter (Chap. 5).

1. Pumps

The primary purposes of pumps are to deliver mobile phase at a precise flow-rate with minimum pulse, and/or to mix the mobile phase for gradient elution. Since the early seventies, piston pumps have dominated the market, supplemented presently by the syringe pump for microbore LC. The syringe pump consists of a piston, driven by a set of gears controlled by a motor. This pump suffers from the major disadvantages of limited reservoir volume and possible solvent "carry-over" contamination. Thus the pump would be stopped prior to refilling the reservoir. A recent innovative application is the integration of the syringe pump into a dedicated analyzer for quality control

or drug assays, such as QA1, as shown by Fig. 2 (Waters Assoc.). By combining the automatic injector, control, detector, and data processing software, such a dedicated system offers attractive features such as space efficiency, minimum personnel involvement, and repair. This concept is new, and it will be interesting to see its acceptability to the clinical laboratories.

Today, the dominant pump is the piston pump. Initially, there was major concern over pulsing problems, but these have been overcome by advances in cam design as well as by use of electronic compensation. The number of pistons varies from one to three, with the smoothest flow from the latter. The advantages include unlimited mobile-phase reservoir, fast mobile-phase changeover, easy maintenance (piston change, pump seal, etc.) and space efficiency. An innovative design by Varian involves coupling to a low-pressure inlet valve, as shown in Fig. 3. This eliminates the need for degassing the mobile phase, a very attractive and time saving feature. Using the piston pump, a gradient can be formed either on the low-pressure side (before the piston) or the high-pressure side (after the piston). Depending on the precision needed, the analyst would have to decide on such a gradient feature. In conclusion, analysts have available a wide spectrum of piston pumps with established reliability and at various price levels (from $500 to $10,000).

Figure 2 QA1 (Reprinted with permission from Waters Assoc.).

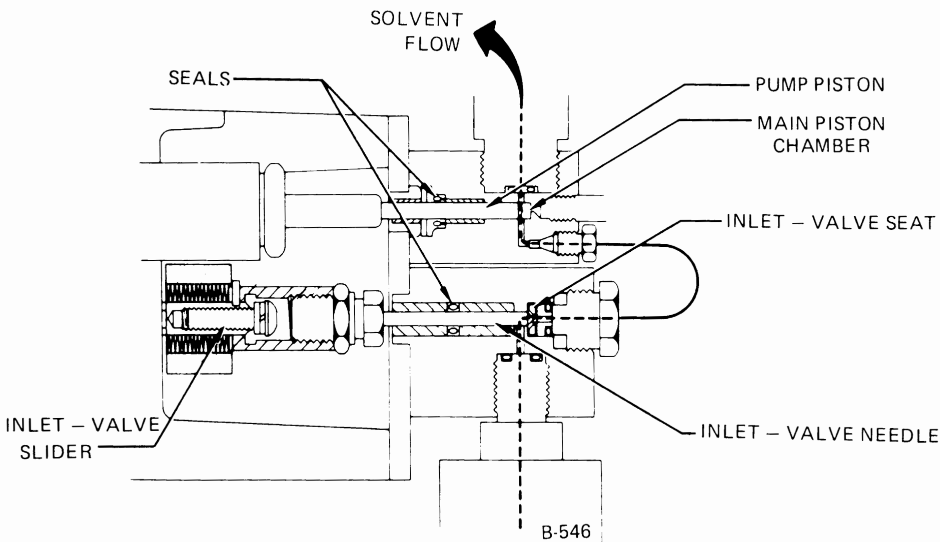


Figure 3 Cutaway view of pump, including detail of pump inletvalve heads. (Reprinted with permission from Varian.)

Our laboratory utilizes all three kinds of piston pumps. Due to rare application of gradient elution in drug assays, low- or high-pressure mixing is not an important factor. The primary considerations are the ease of solvent changeover, the low (pulsing) noise which directly governs the sensitivity of drug assays (picogram or nanogram range), and the easy programming so as to minimize personnel inputting incorrect parameters, and personnel involvement. An important advance is the automated, interactive, statistical technique for LC by Glajch, Kirkland, and Squire [32]. It utilizes computer-designed multisteps for running scouting experiments in arriving at an optimal separation condition within a short time.

2. *Injectors*

The primary requirement for the injector is to introduce sample without a significant contribution to extra-column spreading. The majority of the injectors are continuous-flow injectors with adequate pressure limits (such as Rheodyne, Valvco, and Waters U6K) amply suitable for conventional LC applications. For high-speed and microbore LC, the injection volume would be small. In increasing throughput, an automated injector would be most helpful. Depending upon the needs of the laboratory, most automated injectors would suffice. More sophisticated injectors (from Waters, IBM, and others) incorporate fine features of changing injection volumes at designated vials. For drug monitoring,

an automated injector would certainly enhance the operation of a high work-load drug laboratory, as well as the research and development effort.

3. *Columns*

Due to ease of operation, reliability, and compatibility with biological extracts, reversed-phase has been the most dominant mode of LC used for TDM and toxicology [10,21,23]. In this section, different modes of LC separation will be described, with emphasis on reversed-phase separations and recent developments.

Column packing may be divided into two major categories: non-polymer and polymer. Nonpolymeric packing consists predominantly of silica-based packing, some Al_2O_3, and other materials such as Ca_2SO_4. The silica-based packing accounts for the majority of reversed-phase and normal-phase columns, some ion-exchange and size exclusions packing. Polymeric material, consisting mainly of cross-linked, styrene divinyl benzene, is primarily used for ion-exchange chromatography. A new polymeric reversed packing allows extended pH range (1-13) separation (Hamilton, Rena). For drug monitoring, it may offer selectivity not attainable with conventional silica reversed-phase columns. The reader is referred to other sources for a detailed discussion of column packing [20,22].

Reversed phase: The term "reversed-phase" was coined because the analytes partition readily into the "strong" mobile phase. A typical reversed-phase column consists of silica gel 5 or 10 μm, chemically bonded with a C_{18} chain or other groups (see below). In the presence of a mobile phase such as ACN/H_2O, the analytes (drug molecules) interact with the alkyl chains. Interaction might be based upon a variety of mechanisms such as hydrogen bonding and Van der Waal's interaction. Besides C_{18}, silanol groups may be bonded by C_8, methyl, cyano, phenyl, amino groups, and others. Cyano and phenyl columns are usually classified as normal-phase packing. However, when mobile phase consisting of mixtures of aqueous/organic modifier such as ACN or MeOH are used, the separation mechanism may be better explained as reversed-phase mode. These columns have been used increasingly for drug monitoring [33,34].

Normal phase: The packing consists mostly of silica. Separation is due to the interaction between the "active" silanol groups and the analytes. Due to the long equilibration time, as well as the predominance of reversed phase, normal phase is seldom used in TDM. However, normal phase is very useful in resolving structurally similar molecules, such as in the separation of parent tricyclic and hydroxylated metabolites [35].

Ion exchange: The separation is based on the differences in the interaction with the packing, as governed by the pK_as of analytes.

Compared to reversed-phase, it is not as efficient. However, the water trapped inside the polymeric lattice may induce reversed-phase-like interaction, giving rise to a unique separation mechanism. Besides polymer packing, silica gel has been bonded with ionic functionalities. Application in TDM is limited.

Size exclusion: The modern version of gel permeation separates the molecules according to their size, with the highest molecular weight eluting first. In the ideal situation, all molecules would elute between the void volume, V_0, and the total permeation volume, V_t. Some packings consisting of silica gel with ether functionality have been used extensively for protein separation. Possible applications in TDM and toxicology await exploration.

Miscellaneous: In the separation of ionic species, ion pairing has been used to offer separation not possible with a straight reversed-phase mode [31]. Specialized columns such as the Z-module (Waters Assoc.) offer extremely efficient separation with low back-pressure and easy column changeover. Screw-on column cartridges (Brownlee Lab.) offer snap on/off ease.

4. Mobile Phase and Other Practical Considerations

For detailed discussions of mobile phase, the reader is referred to the following references: [10,17,21,38]. Generally, a reversed-phase mobile phase for TDM/toxicology consists of an aqueous component and an organic modifier (5-40% for most applications). A third component such as THF or ion-pairing reagent is occasionally added. Phosphate mobile-phase should be refrigerated to avoid contamination. For low-UV detection, HPLC-grade water is highly recommended. Daily operation should include a "cleanup" of the column with 10-15 column volumes of water and ACN after drug measurements. The percentage of ACN should be higher than the mobile phase in order to elute previously retained components.

A dedicated column for each drug or group of drugs with similar chemical structure would enhance the operational efficiency. It may be achieved by (a) using a different column for each assay, (b) using switch valves to minimize tightening and loosening of the columns, (c) using a dedicated chromatograph or analyzer such as QA1, and (d) using radial compression modules, Z modules, or screw-on columns. With the above approaches, equilibration time is short, usually 10-30 min, and reliability is assured.

5. Detectors

Detectors may be divided into two kinds: bulk and specificity. Since the majority of drugs are organic and have a conjugated double bond, the most popular detector for TDM and toxicology is the ultraviolet

(UV). Other detectors of limited use are fluorescence, electrochemical detectors, and mass spectrometers. In this section, recent advances in UV detection are described, followed by a brief discussion of the less familiar but useful photoconductivity detector along with the radioactivity detector. Other important detection methods will be discussed in later chapters on electrochemical detection, the mass spectrometer and LC-MS interface, and on fluorescence detection and associated techniques, including derivatization.

Ultraviolet detectors: For TDM and toxicology, the most popular detectors are fixed-wavelength detectors with 214-, 254-, or 280-nm filters. Flow-cell volume is about 10 μl or less to reduce extra-column speading. Due to tapered flow-cell design of the Model 440 detector (Waters Assoc.), the refractive index effect is minimized. With its enhanced sensitivity, it might obviate the need for variable-wavelength detection, even though the wavelength of the detector does not coincide exactly with the drug absorption maxima.

Following the fixed-wavelength detector, variable-wavelength detectors are considered essential second detectors for the clinical laboratory. Some detectors perform wavelength ratioing and/or scanning for possible drug identification. Previously the scanning operation was performed with a stop-flow technique. Two recently introduced, variable-wavelength detectors capable of rapid scanning of elution peaks are the Model HP1040A (Hewlett-Packard) and the Model 165 (Beckman). Model HP1040A makes use of photodiode array photocells embedded onto a silicon chip, capable of monitoring from 190 to 600 nm simultaneously in milliseconds. The deuterium lamp radiation, focused by an achromatic lens system, passes through a flow cell. The emergent radiation is dispersed by a diffraction grating onto the photodiode array, coupled to a computer capable of processing and storing the data.

The Model 165 detector utilizes a magnetically suspended mirror. Wavelength ratioing or scanning may be achieved by rapid changes in the magnetic field. Such a spectrum has been performed for the tricyclic antidepressant nortriptyline (Fig. 4).

With these new developments, UV detection has been firmly established as a choice detector for TDM and toxicology assays. Wavelength ratioing and scanning, however, would provide useful but not definitive information on the identification or purity of the peak.

Fluorescence: Due to its higher sensitivity and the possibility for derivatization, fluorescence detection has been used for certain drugs such as antidepressants [35] and antihypertensives [36]. A detailed treatment of the principle related to this technique and examples will be provided in Chap. 7 by Weinberger.

Electrochemical detector: Like fluorescence detectors, electrochemical detectors have been useful in certain clinical assays, such as for cate-

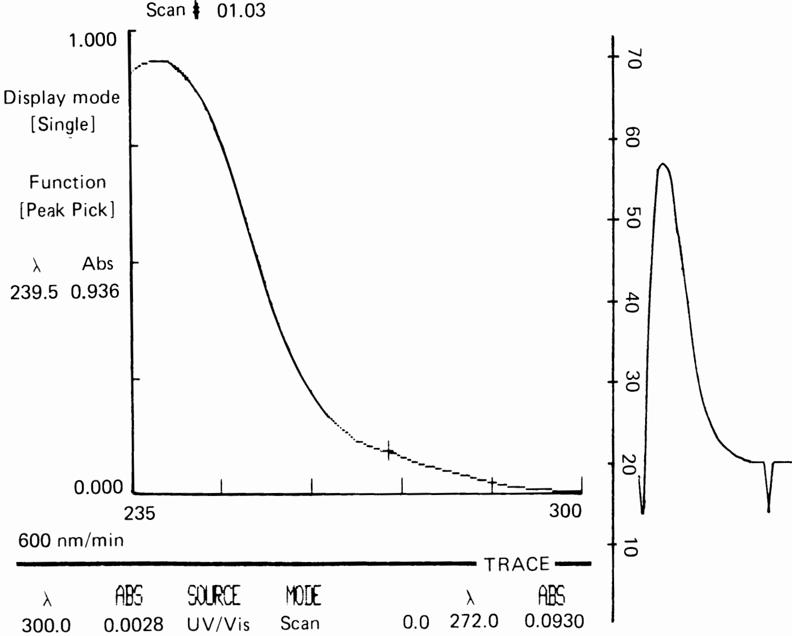


Figure 4 (Left) Spectrum of nortriptyline dissolved in mobile phase, using the DU-7 scanning spectrophotometer (Right) "On-the-fly" scan of peak eluting through the flow-cell of Model 165 detector (Beckman Instrument) (max = 240 nm). (Courtesy of Mr. Cogan of Beckman Instruments.)

cholamines. The principle of LC-EC and applications for TDM and toxicology will be included in the chapter by Kissenger and Lavrich (Chap. 8).

Mass spectrometer: Like GC-MS, this technique provides the ultimate identification. LC interface with the mass spectrometer—the approach, the problems, and the application for TDM and toxicology—will be discussed in the chapter by Eckers and Henion (Chap. 6).

Photoconductivity: This technique provides selective detection for compounds containing halogen, nitrogen, and sulfur [39]. The principle is based upon the formation of ionic species as a result of photolysis, followed by their conductivity measurement, as proposed by Hall and Rogers.

In order to minimize ionic interference, ion-exchange resin is integrated with the regular LC pumping system for removing ionic species. The mobile phase enters a reaction system and is split into

two streams. One stream enters a reactor coil and is exposed to a 214-nm zinc lamp or 254-nm mercury lamp for photolysis; the other stream enters a delay coil. Both streams enter the detector differential conductivity cell. The signal is processed by the detector.

Figure 5 shows a comparison of UV absorbance and photoconductivity repsonse of sulfonamides. Picogram sensitivity may be ob-

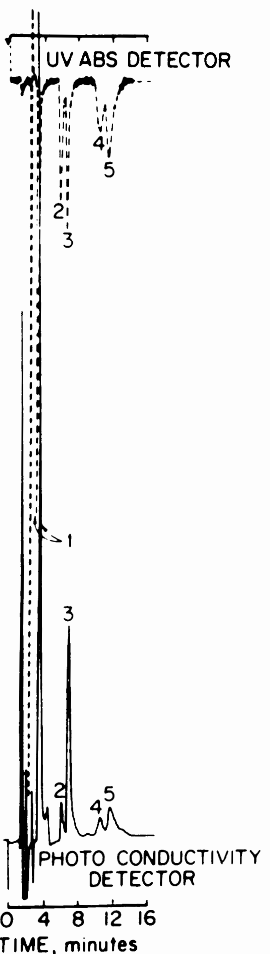


Figure 5 Chromatograms of sulfonamides. Several response differences can be seen in this sulfonamide separation. Peak identification: 1 = sulfanilimide; 2 = sodium sulfadiazine; 3 = sulfathiazole; 4 = sulfabenzidine; 5 = sulfamethazine; 25 ng each compound. A Zorbax ODS column was used with an eluent of 85:15 acetonitrile and water at a flow-rate of 1 ml/min. (From Ref. 39.)

tained for some of these compounds. The detector is compatible with both reversed- and normal-phase LC. In combination with a UV detector, it may enhance the identification of drugs and toxic chemicals.

Radioactivity detectors: Radioactivity detectors are not used for TDM and toxicology. However, for pharmacokinetic and pharmacological studies, they are quite valuable. The radioactivity detectors are classified into two major kinds according to the decay modes: beta and gamma particles. For the detection of gamma radiation, a scintillation crystal with the appropriate energy setting may be used [40]. The output, corresponding to counts per unit time, is then recorded. Gamma detection is easy and requires minimum technical consideration. In contrast, for beta detection of low energy-emitting radionuclides such as tritium, and because of associated quenching, quantitative detection is more difficult. A recent paper has reviewed the status of radioactivity detection in chromatography [37]. Progress has been made, but problems still exist.

Miscellaneous: Refractive index and infrared detectors are seldom used for TDM measurements.

5. *Data Processing Systems*

The availability of the laboratory and personal computer has revolutionized the data processing process. Since most LC drug assays show a linear response between concentration and peak height and/or peak height ratios (with internal standards), a simple strip chart recorder would suffice. However, as the volume of chromatograms increases rapidly, the computerization of data for evaluation, storage, and retrieval becomes important. Old data may be reanalyzed to check items such as interference and column stability. Since TDM and toxicology data may be used in medicolegal situations, accuracy of data cannot be overlooked. The application of computers is discussed in the chapter by Scott (Chap. 5).

In optimizing sample preparation and analysis, Snyder et al. described an automated system, "FAST-LC" [42], which unfortunately was discontinued recently. However, such a system, as shown in Fig. 6, represents the ultimate in LC automation for drug assay. Also, the application of commercially available extraction cartridges and apparatus such as Bond-Elute (Analytichem), Sep-Pak (Waters Assoc.), and Prep-I (Dupont) represent important advances in sample preparation. This approach is increasingly used in clinical laboratories.

In conclusion, LC has offered highly selective and fast analytical methods for TDM/toxicology assays. The application depends on many factors, each of which weighs differently in different laboratories. Besides obvious technical factors, such as a good LC assay compared to GC and other immunoassays, other no-so-obvious factors include

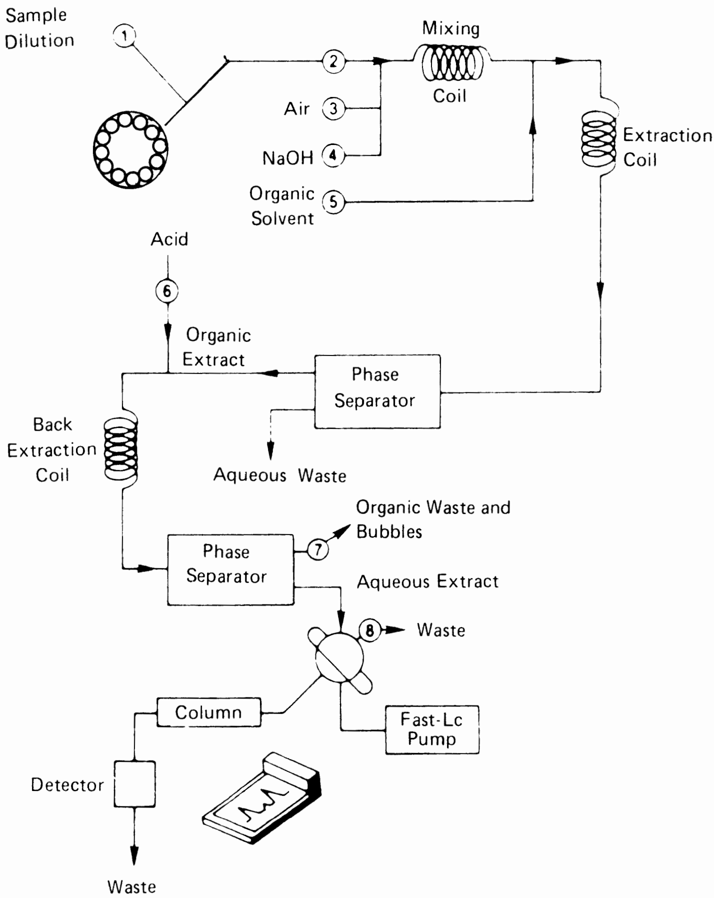


Figure 6 "FAST-LC" for tricyclic analysis. (From Ref. 42.)

the training of the laboratory personnel, especially the director, the availability of resources (funding, instrument space, personnel, etc.). The author believes that LC will always be a vital part of TDM and toxicology.

III. GAS CHROMATOGRAPHY

Gas chromatography has been vitally important for therapeutic drug monitoring and toxicology, and GC-MS provides the definitive structural identification. This section discusses briefly the gas chromatographic process and recent developments pertinent to TDM and toxi-

cology. For a complete and updated treatment on principle, instrumentation, and applications, readers are referred to the following sources: [9,12,24,41,43-46].

Gas chromatographic separation is based upon the partition of the analytes with the stationary phase. The analytes are usually vaporized at elevated temperatures. Carrier gas is used for transport, but does not directly affect the partitioning procedure, as in the case of the mobile phase in LC. Derivatization may be needed to render the analytes more volatile. Injectors, ovens, and detectors are discussed in Refs. 9, 12, 24. Werner [11], and Toseland and Wicks [12] have outlined the various columns for drug monitoring. Silanized columns may be used to minimize basic drug adsorption. Besides the popular flame ionization detector, alkaline flame detectors are increasingly used for the detection of drugs containing nitrogen and phosphorus. Due to the high resolution of capillary columns, drug screening and other measurements may be carried out with increased efficiency and sensitivity. Recently, simplified mass spectrometric detectors such as the mass selective detector (Hewlett-Packard) and ion trap detector (Finnigan) were introduced. Supposedly, both detectors are easily adapted to clinical drug identification, because of their compact design and their interface with a limited drug identification library. Another detector of increasing interest is the FT-IR for GC [46].

In summary, GC has played an important role in TDM and toxicology. With the advent of capillary columns and mass selective and ion trap detectors, the role of gas chromatography in clinical laboratories may be redefined. Compared to LC, however, GC drug assays always involve lengthy sample preparation/extraction/derivatization procedures. Moreover, elevated-temperature separation may decompose some drug molecules. GC, however, will remain a complementary and vital tool in TDM and toxicology.

IV. RADIOIMMUNOASSAY (RIA)

Radioimmunoassay (RIA) has played a very important role in the clinical sciences. Introduced by Yalow and Berson [47], the technique has extended the detection limits and has offered specificity for biological assays. Digoxin quantification by RIA was one of the early applications [12]. Since then, a number of RIA drug assays have appeared, such as those for phenytoin, phenobarbital, procainamide, N-acetylprocainamide, methotrexate, gentamicin, vancomycin, amikacin, and others. However, these RIA assays are not as extensively used as that for digoxin, possibly due to the availability of other ligand-binding assays, such as enzyme-multiplied immunoassay techniques (EMIT), introduced in the seventies. Recently, an RIA assay for the antidepressant nortriptyline was introduced. Progress in the applicability of RIA for drugs, however, has been limited, but un-

questionably, RIA generated the impetus which resulted in the modern forms of ligand binding assays. Thus an elementary understanding of the RIA principle would be desirable and is outlined as follows [48]:

$$L^* + L + Ab \rightleftharpoons \underbrace{LAb + L^*Ab}_{\text{bound}} + \underbrace{L^* + L}_{\text{free}} \qquad (7)$$

where

L^* = the radiolabeled ligand or bound tracer,

L = the unlabeled ligand, or the patient drug molecules, and

Ab = the antibody.

In the presence of limited binding sites of the antibody, L^* and L undergo competitive binding. After reaching equilibrium at the end of the incubation period, L and L^* will form LAb and L^*Ab, the "antibody-bound" fraction, while the remaining L and L^* will remain as the "free" fraction. It is desirable to utilize antibodies with high avidity towards the ligands, so that equilibrium can be quickly reached. De-

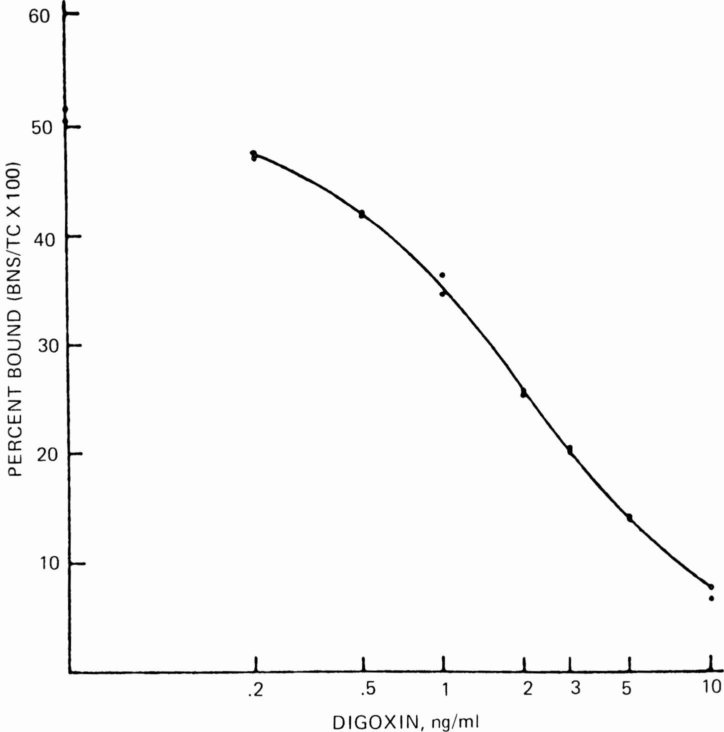


Figure 7 Digoxin RIA calibration curve. (Reprinted with permission from *Clinical Chemistry*, Ref. 49.)

pending upon the amount of L (patient drug), a corresponding amount of L* will be displaced from the antibody fraction.

For a sample with low drug concentration, the antibody-bound fraction will consist mostly of the L*Ab fraction; conversely, a high-concentration sample will result in a large LAb fraction, displacing L* from L*Ab. The subsequent radioactivity in the free fraction will be high. Figure 7 shows the percent bound vs. free drug concentration for digoxin [49]. Most clinical drug assays such as that for digoxin may be readily carried out with excellent precision within several hours by using commercially available kits.

In developing an RIA, the considerations include producing antibodies with high titer, avidity, and specificity. Tracers should incorporate gamma emitters with high specific activity, such as ^{125}I or ^{75}Se. However, a pressing problem is radioactive waste disposal, which has certainly circumscribed the various applications of radioactive nuclides, such as RIA and radiopharmaceutical production.

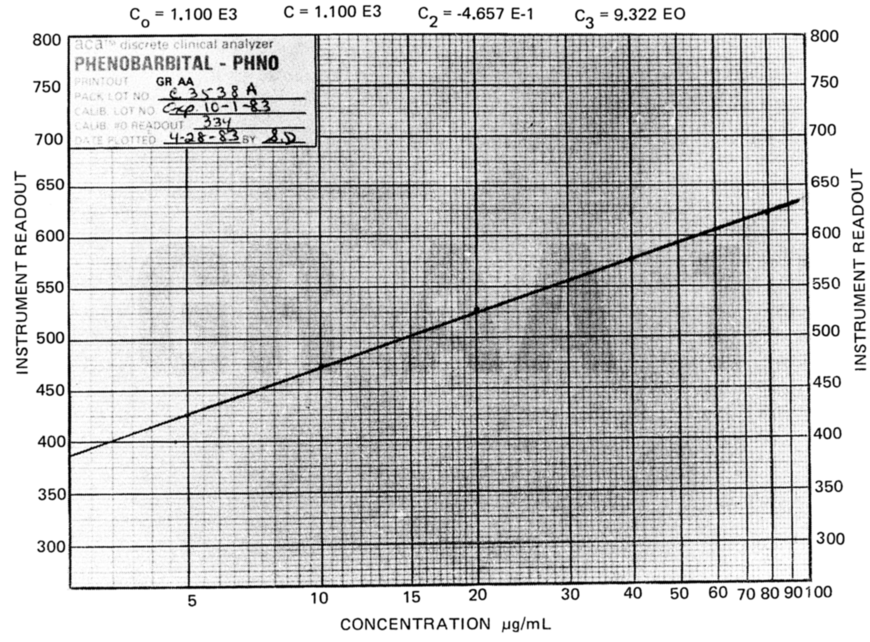
V. ENZYME MULTIPLIED IMMUNOASSAY TECHNIQUES (EMIT)

Rubenstein et al. [50] first introduced a homogeneous enzyme immunoassay. The term "homogeneous" refers to ligand assays which do not require a separation step for the ligand and ligand-antibody complex. Shortly after this report appeared, the screening of urine samples for drugs of abuse and for monitoring antiepileptics [51] by EMIT was introduced by Syva Co. (Palo Alto, California). Since then, EMIT assays for other groups of drugs as well as endogenous metabolites (T_3 and T_4) have been introduced, offering a wide range of assays with applications in clinical chemistry, TDM, and toxicology. Since they can be performed quickly by a technologist with minimal training, EMIT drug assays have been the dominant and most widely used method. Their popularity has been further enhanced by automation. Four extensively used antiepileptic assays—for phenobarbital, phenytoin, primidone, and carbamazepine—are carried out by the autoanalyzer, Automated Clinical Analyzer (Dupont, Wilmington, Delaware). Other autoanalyzers adaptable to EMIT include COBAS-BIO, VP, and others [52-55]. Recently, additional analyzers, known as the Autocarousel and QST, which use dry powder reagents, were introduced by Syva to enhance the use and/or the shelf life of reagent. Both utilize the same EMIT principle which is based upon the measurement of enzymatic activity [65]. The enzyme, glucose 6-phosphate dehydrogenase (G6PDH), ED 1.1.1.49, is bound to a drug such as phenobarbital to form a conjugate without changing the enzymatic activity. However, upon binding to anti-drug antibody, the resultant steric hindrance causes a decrease in the enzymatic activity of the conjugate.

The assay is performed by mixing patient plasma or serum containing drug molecules such as phenobarbital. These molecules and

the conjugate undergo competitive binding for the antibodies to form either drug- (phenobarbital) antibody complexes or enzyme conjugate-antibody complexes. Such complexation of the enzyme conjugates results in steric hindrance of the enzyme active sites, reducing the enzymatic activity in proportion to the amount of phenobarbital molecules in patient serum. Reduction in enzyme activity is monitored in the presence of a substrate and the cofactor nicotinamide adenine dinucleotide (NAD). Thus the reduction of NAD to NADH is lowered, and the rate of reduction is measured by the absorbance change at 340 nm. Since the G6PDH enzyme used with the EMIT procedures is derived from the bacterium *Leuconostoc mesenteroides*, the cofactor does not interact with endogenous G6PDH. The change in absorbance, A, vs. log drug concentration (in this case, phenobarbital) is shown in Fig. 8. Patient serum drug levels are estimated from this plot.

As with any ligand assay, the manual procedure requires careful pipetting techniques and precise timing for competitive binding and measurement. Other considerations include the buffer, which is specific for a particular group of drug assays; temperature control; premeasurement warm-up time (half-hour); and the measurement tem-



perature (flow-cell temperature kept at 37°C). Some of the parameters are well controlled by instruments such as the diluter and clinical processor (timer, data collector, and a calculator). Possibly due to cross-reactivity with metabolite(s) of phenytoin, present only in samples from renal patients, Bowers et al. [56] observed elevated EMIT measurements of both free and total phenytoin.

Recently, a modification of this enzyme immunoassay using fluorescence detection was introduced as the Advance automated analyzer (Syva) and is shown in Fig. 9. The principle is based on fluorescence quenching which occurs as a result of the binding of the fluorescent-labeled analyte to the quencher antibody. Drug molecules such as phenobarbital from patient serum and the fluorescent-labeled analytes compete for antibody binding, resulting in the quenching of the fluorescence of the labeled analyte. Like other EMIT responses, the quenching rate is dependent upon the analyte concentration. Other advantages include the small sample volume (as little as 6 μl), the input of patient identification, the storage of calibration curves, and calculation of data.

In conclusion, EMIT has been the most widely used nonradioactive immunoassay for TDM and toxicology. New instrumentation such as Auto-Carousel, Advance, and QST are important advances. It will be interesting to establish the clinical efficacy of these techniques.

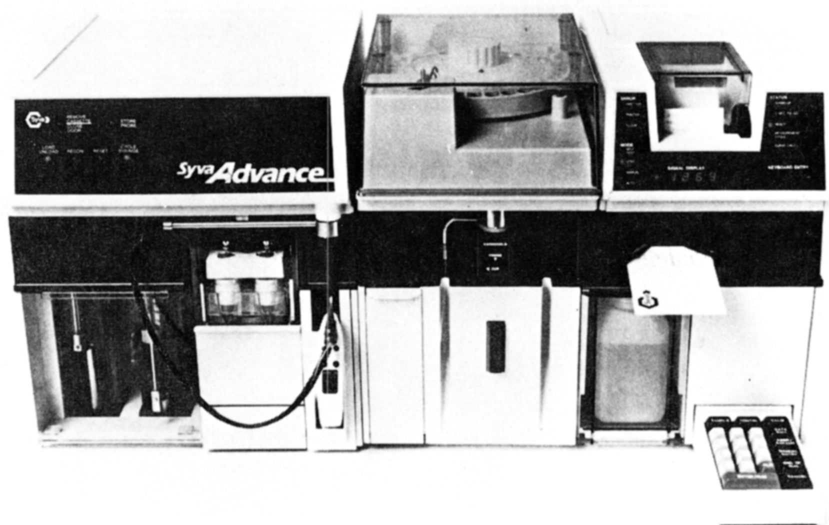


Figure 9 Advance autoanalyzer by Syva.

VI. FLUORESCENCE POLARIZATION IMMUNOASSAY (FPIA) USING TDX

TDX, an automated clinical analyzer, based on FPIA, was introduced 2 years ago (by Abbott Labs.) for TDM and toxicology measurements. The technology has been well received by the clinical laboratory community. In this section, the principle and selected applications will be described. The principle is based upon fluorescence polarization, initially described by Perrin [57], and on competitive binding [58,59]. Dandliker applied the technique for biological systems and for the measurement of antigen-antibody interaction [58]. Jolley discussed the theory, the technical problems, and some clinical applications [59, 60]. Blecka [61], Bakerman [62], and Wong [63] recently reviewed the technique.

The original problems included inappropriate instrumentation, nonspecific binding of the tracer, and fluorescence interference of the serum. These difficulties were overcome by use of the microprocessor-controlled analyzer for measurement and data processing, the production of high-affinity antibodies, and the use of a small sample to reduce endogenous interference. The instrument is shown in Fig. 10, and the major components in Fig. 11.

According to the Perrin equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + 3\,\frac{\tau}{s}\right) \tag{8}$$

where

P = the observed polarization of fluorescence,

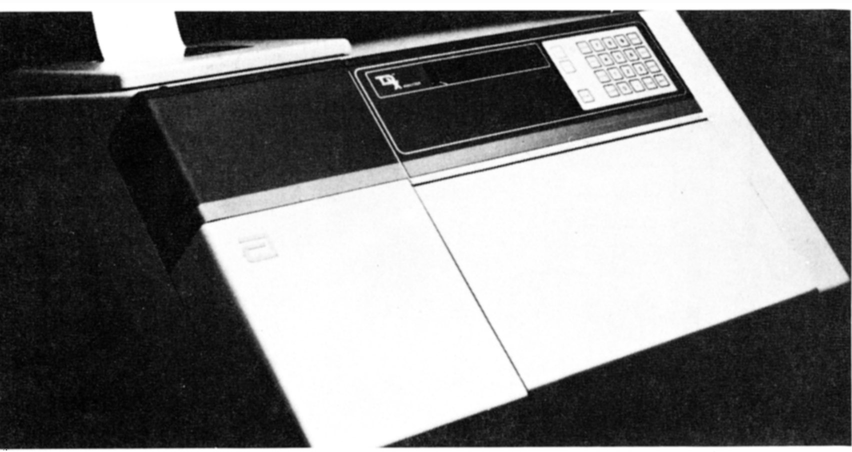


Figure 10 TDX autoanalyzer by Abbott.

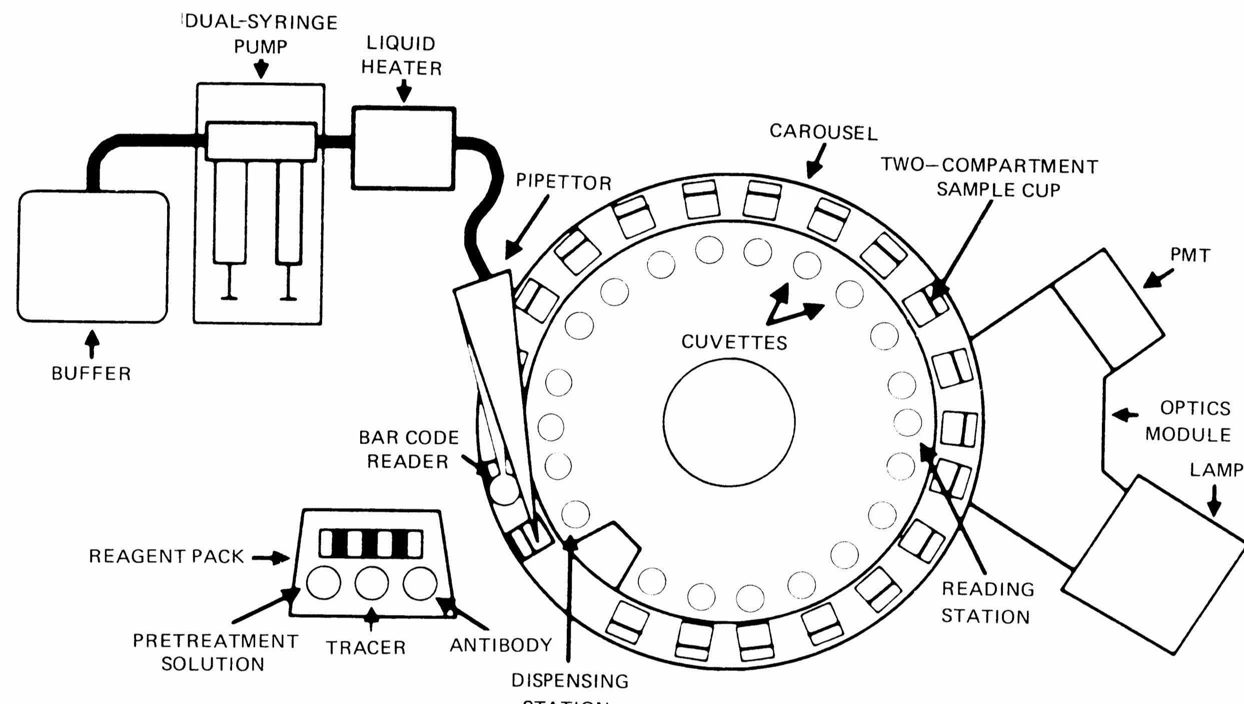


Figure 11 The major components of the totally automated, bench-top fluorescence polarization analyzer (PMT, photomultiplier tube). (Reprinted with permission from *Lab. Mgmt.* *22*(7), 37 (1984).)

P_0 = the limiting polarization of fluorescence,

τ = the fluorescence lifetime of the molecule, and

s = the rotational relaxation time of the molecules.

For a group of dipoles parallel to plane-polarized light, these dipoles, or fluorophores, absorb and then emit polarized light parallel to the incident light. If the fluorescence lifetime, τ, is constant, the degree of fluorescence polarization depends on s, which is influenced primarily by the molecular size. Thus fluorescence polarization is likewise directly related to the molecular size. This phenomenon, which when coupled with competitive binding is termed FPIA, and is used for drug measurement as follows: The tracer drug, or a similar chemical labeled with fluorescein (F-Ag), and patient serum drug molecules (Ag) compete for the binding sites of the antibodies as shown:

$$\text{F-Ag} \quad \text{Ab} + \text{Ag} \rightleftharpoons \text{F-Ag-Ab} \quad \text{F-Ag} + \text{Ag-Ab} \quad \text{Ag}$$

When exposed to the incident plane-polarized light, the polarization of fluorescence is increased due to slow rotation of the large tracer-antibody complex. This fluorescence polarization is effectively measured by sequential excitation with vertically and horizontally plane-polarized light. The change is governed by preset voltages applied to a liquid crystal to filter the incident light. Polarization of fluorescence, P, is estimated as follows:

$$P = \frac{I_{vv} - I_{hv}}{I_{vv} + I_{hv}} \qquad (9)$$

where I_{VV} is the intensity of the vertical component of the emitted light (525-550 nm), caused by vertically polarized incident light (485 nm); and I_{hV} is the intensity of the vertical component of the emitted light, caused by horizontally polarized incident light.

Thus a patient specimen with low analyte (drug) concentration will result in a large amount of tracer (F-Ag) bound to antibody, with subsequent high polarization of fluorescence. And conversely, specimens with low analyte concentration will result in low polarization of fluorescence. The relationship of polarization of fluorescence to analyte concentration (such as that of valproic acid) is shown in Fig. 12. This information is stored in the microprocessor for each drug assay for up to 4 weeks. During a clinical assay, patient serum drug concentrations are automatically estimated from the stored calibration data.

The procedure is simple and efficient: Add aliquots (50 μl minimum) to the sample well of the cartridges, placed inside a marked

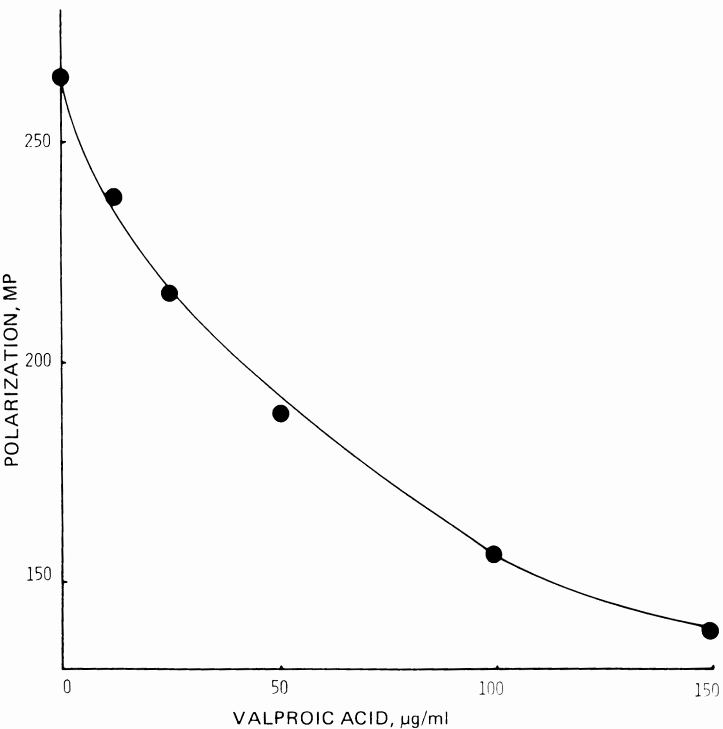


Figure 12 FPIA calibration curve for valproic acid.

carousel with the corresponding number of curvettes. Place the reagent kit inside the instrument. Press RUN on the instrument panel. The barcode reader scans the reagent kit. TDX performs self-diagnostic tests such as temperature and reagent levels. The microprocessor then sets the appropriate parameters. The procedure is then carried out in four revolutions: first revolution, sample dilution with buffer; second revolution, pretreatment of part of the diluted sample/buffer in the cuvette, followed by "blank" fluorescence measurements; third revolution, addition of tracer, antibodies, and additional diluted sample and incubation for 3 min; and fourth revolution, measurement of fluorescence polarization. A typical printout for valproic acid is shown in Fig. 13. Figures 14-16 present correlation studies of valproic acid, theophylline, and lidocaine with an established method or survey target values. These data showed acceptable correlation [63].

The innovative features include the application of a liquid crystal for generating polarized light, a universal buffer for all drug assays

DATE 08/01/83
TIME 14:40:39
ASSAY: VALPROIC ACID

CALIBRATION

VOL= 1.00
REPS= 2
GAIN= 40

CONC= UG/ML

I.D.		NET P	NET I	BLANK I
1	A	266.44	6709.4	594.8
2	A	266.73	6962.7	560.0
3	B	237.11	6891.8	587.6
4	B	238.02	6958.5	585.3
5	C	216.10	7110.2	544.6
6	C	215.73	6992.6	542.3
7	D	187.87	7223.9	558.7
8	D	188.69	7360.7	542.8
9	E	156.83	7750.6	556.9
10	E	157.84	7647.4	572.7
11	F	138.55	7882.5	554.0
12	F	139.43	7966.3	571.0

A= 59.54 B= 11857.69
C= 60.18 D= 0.94
ITR= 2

I.D.	CONC	AVGP	FITP	PERR
A	0.00	266.59	266.58	0.01
B	12.50	237.57	236.73	0.84
C	25.00	215.92	216.31	- 0.39
D	50.00	188.28	188.45	- 0.17
E	100.00	157.34	156.71	0.63
F	150.00	138.99	138.79	0.20

RMSE= 0.41 DF= 8

SAMPLES

LOC	CONC	NET P	BLK I
13	47.88	190.36	570.05
14	88.93	162.10	556.43
15	45.89	192.22	558.73
16	100.16	156.63	570.08
17	38.21	200.00	535.60

Figure 13 FPIA calibration and patient data printout. (Reprinted with permission from *Lab. Mgmt.* *22*(7), 38 (1984).)

facilitating assay throughput, temperature stability at 35 ± 0.5°C, the long shelflife of the reagent kit (with the exception of vancomycin calibrators), and "STAT" capability. Recently, Elin reported discrepant theophylline measurement of serum from a renal-failure patient [64].

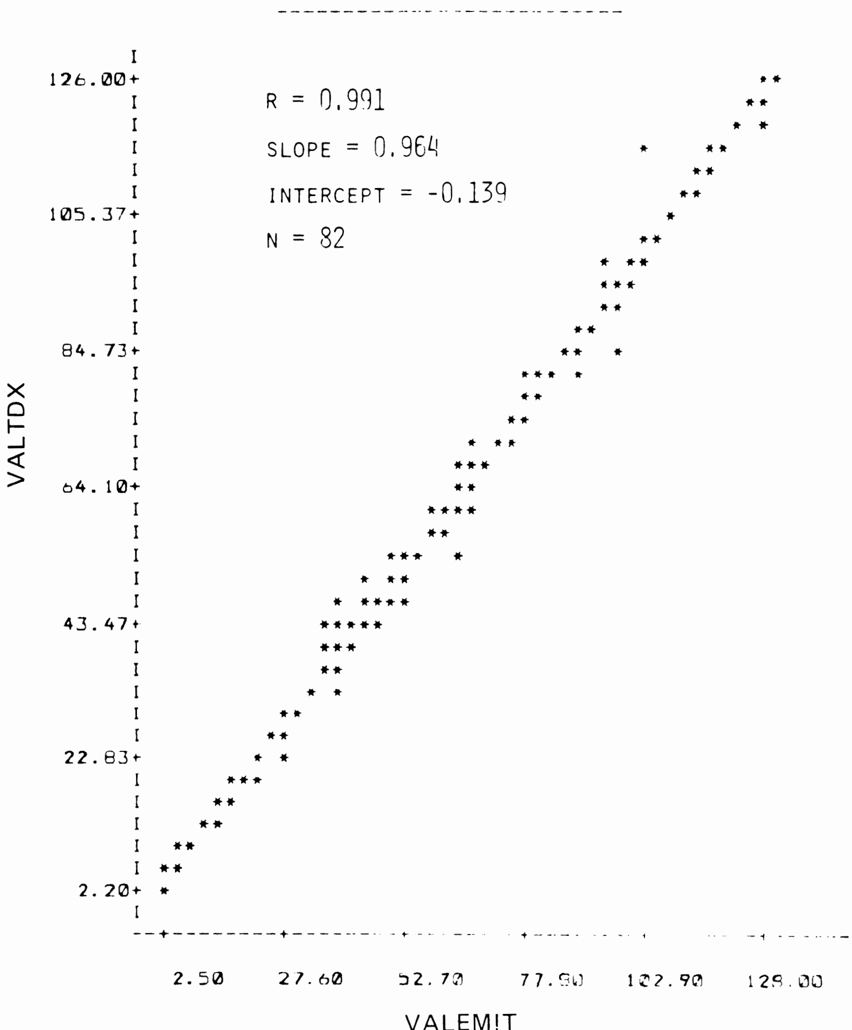


Figure 14 Correlation of FPIA (y) with EMIT (x) measurements of valproic acid. (Analyzed by the Correlation and Linear Regression Analysis Program, Advanced Statistical Analysis, Radio Shack, Fort Worth, TX.) Dots represent both data points and regression line. (Reprinted with permission from *Lab. Mgmt.* *22*(7), 39 (1984).)

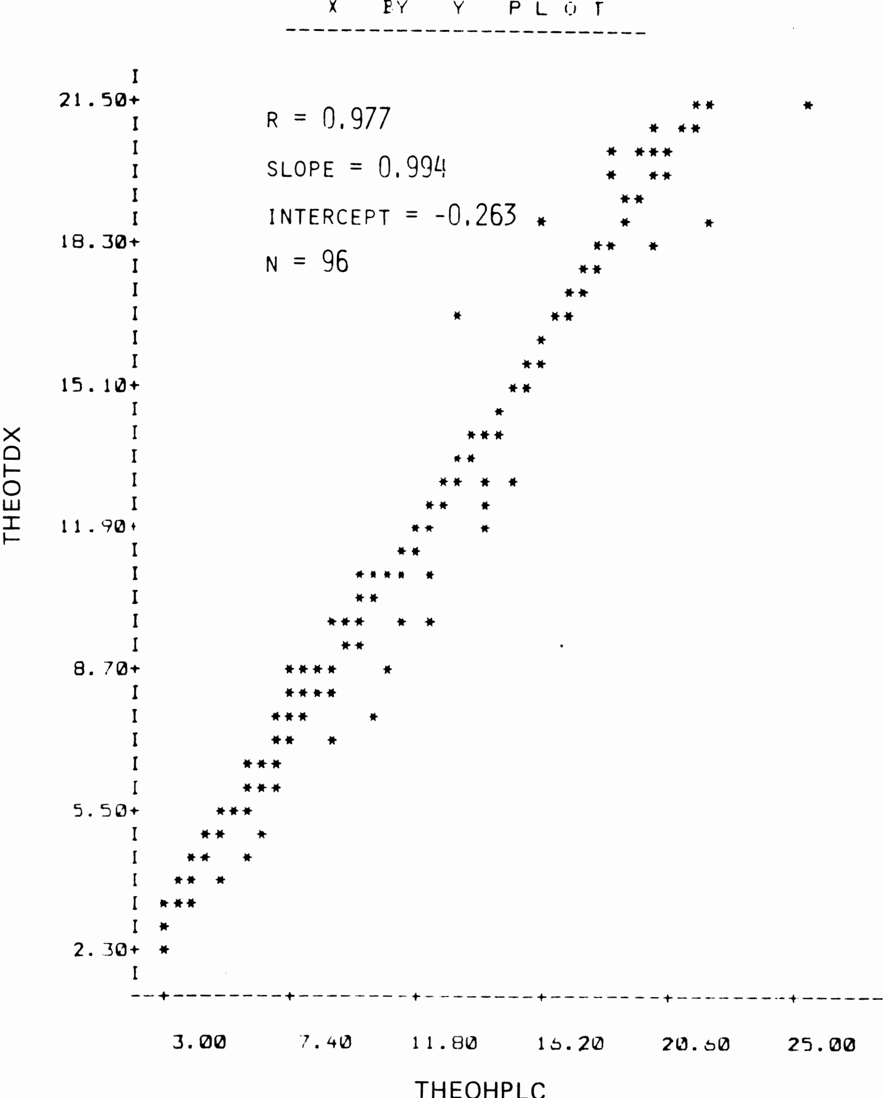


Figure 15 Correlation of FPIA (y) with HPLC (x) measurements of theophylline.

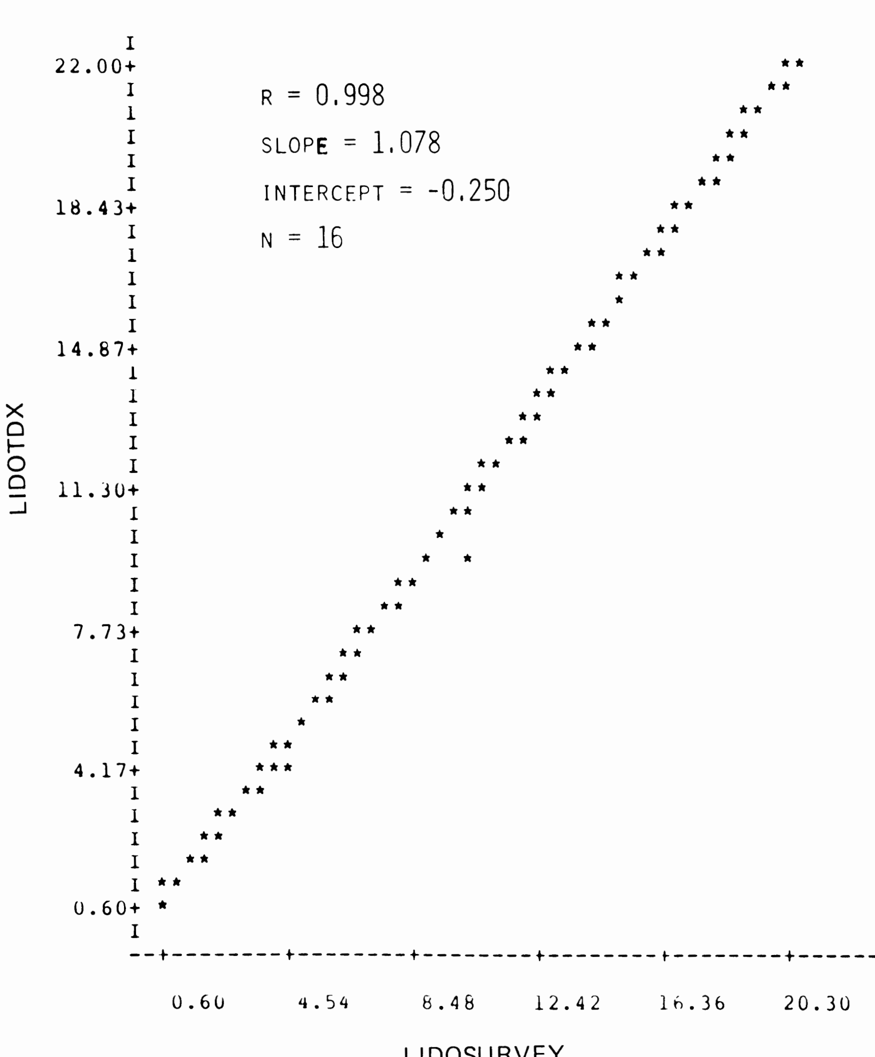


Figure 16 Correlation of FPIA (y) with target values (x) of lidocaine of AACC and CAP surveys.

VII. SUBSTRATE-LABELED FLUORESCENCE IMMUNOASSAY (SLFIA)

The principle of SLFIA (Ames Laboratory) is based on competitive binding and the β-galactosyl-umbelliferone-sisomicin conjugate enzymatic

reaction of the fluorogenic drug gentamicin reagent, FGR [65] (Fig. 17). The FGR and patient serum drug molecules, such as gentamicin, compete for antibody binding sites. FGR-Ab complex is nonfluorescent. In the presence of β-D-galactosidase, the remaining free FGR is hydrolyzed to form a fluorescent product, unbelliferone-sisomicin. The timings for the addition of FGR and for the measurement of fluorescence must be precisely controlled. For low drug concentration samples, FGR is bound mostly to the antibody with minimum free FGR remaining. Thus the resultant fluorescence intensity is low. Conversely, for high drug concentration samples, fluorescence intensity is high.

Besides TDM measurement, SLFIA has also been used for measuring blood chemistry. An automated version, named Optimate, shown in Fig. 18, was introduced recently, with microprocessor control for the pipetting, measurements, and data manipulation. Reagent kit is claimed to be stable up to 12 months. However, from a recent report, nonlinearity of the calibration remains a problem for the gentamicin assay [66]. Recently, a theophylline assay which uses monoclonal antibody was introduced, probably providing specificity important for monitoring renal patients [63].

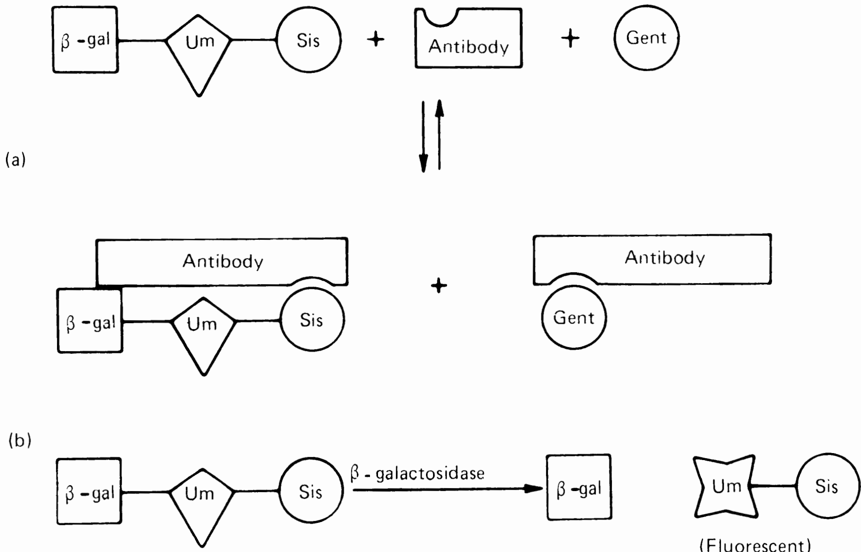


Figure 17 Schematic of the reaction sequence for the fluorescent immunoassay of gentamicin. (From Ref. 66.)

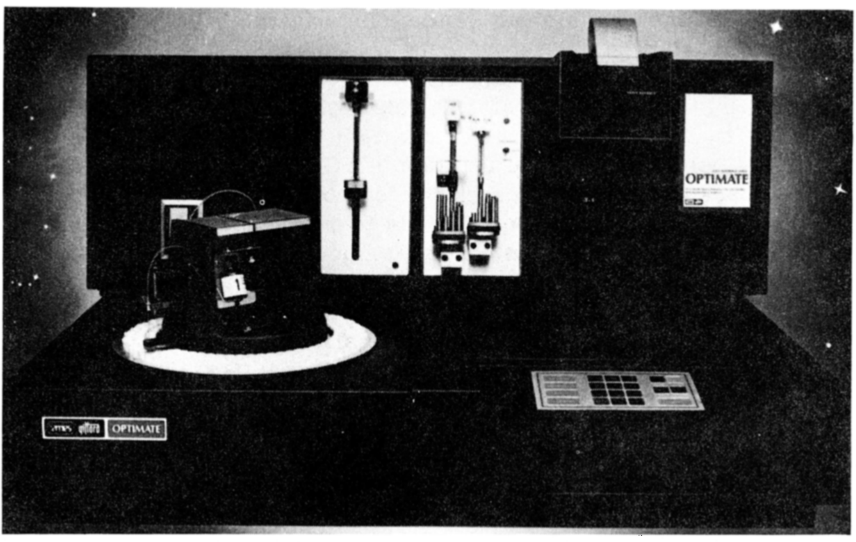


Figure 18 Optimate autoanalyzer by Ames. (Courtesy of Ames Laboratories.)

VIII. NEPHELOMETRIC INHIBITION IMMUNOASSAY (NIIA)

Nephelometry is the measurement of scattering and/or reflected light by a detector located outside the path of the transmitted light. Using the Immunochemistry System, ICS, from Beckman Instruments, a number of nephelometric assays for drugs have been developed.

Pauling is credited for the initial nephelometric inhibition immunoassay assay [67]. The principle of NIIA (Fig. 19) is based upon competitive binding between the conjugate and the hapten (drug) for antibody [68-70]. Conjugate is synthesized by covalently linking the hapten to a carrier protein. These conjugates bind with the antibodies (Ab-conjugate) to form light-scattering centers. In the presence of the hapten or drug molecules, Ab-drug soluble complexes are formed, hindering the formation of light-scattering Ab-conjugate centers. Thus the light-scattering intensity is lowered. The nephelometric assay by the ICS system is rate dependent. An automated version has been introduced recently. In contrast to FPIA, automated NIIA makes possible simultaneous, multidrug analysis, and allows interruption for STAT testing.

IX. LATEX AGGLUTINATION INHIBITION TEST FOR GENTAMICIN AND TOBRAMYCIN

This immunoassay, marketed by Becton-Dickinson under the name of Macro-Vue^{TM} card test for gentamicin and tobramycin, is based on a

(a)

Conjugate + Antibody

(b)

Conjugate + Antibody + Hapten

Figure 19 Principle of nephelometric inhibition immunoassay. (a) Conjugate-antibody complexing. (b) Inhibition of complexing by hapten. (From Ref. 69.)

competitive agglutination reaction between patient serum or plasma gentamicin and gentamicin-sensitized latex particles for anti-gentamicin antisera [71]. Patient gentamicin molecules inhibit the agglutination. The patient's gentamicin concentration is estimated by comparing the inhibition of various diluted specimens with the standards. Figure 20 shows that from the card, the patient plasma gentamicin concentration was estimated to be 8 mg/L. The assay was compared to both microbiological and RIA assays with favorable results. Because of the ease of performance, this may be a good procedure for STAT after-hour assays.

X. ENZYME-LINKED IMMUNOSORBENT ASSAY: ELISA ENZYMUNE-TEST™

ELISA, a heterogeneous immunoassay, marketed by ICL Scientific, has been developed for digoxin. The principle is based on the competitive

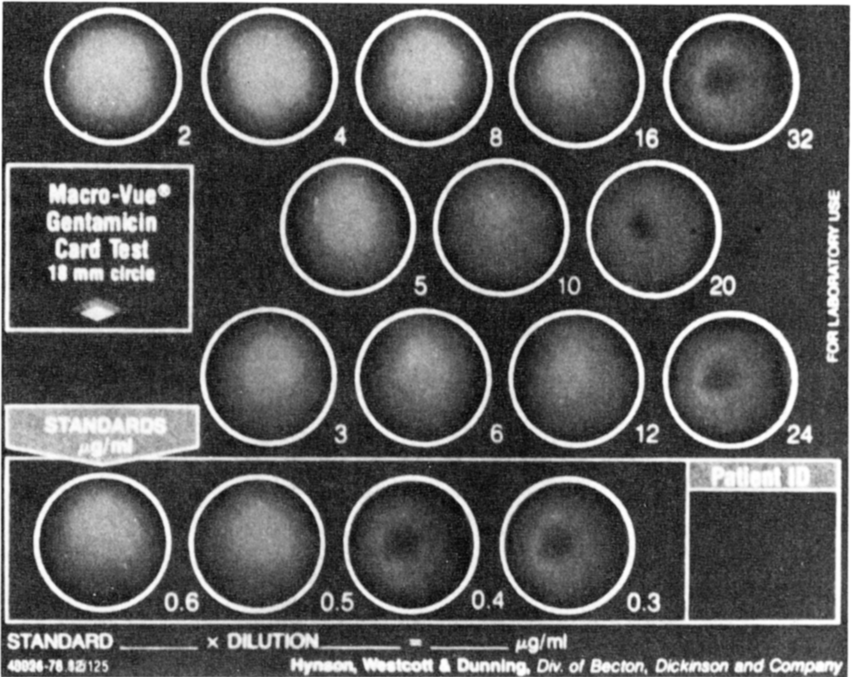


Figure 20 Macro-Vue™ gentamicin card test. (Courtesy of Becton-Dickinson.)

binding of patient plasma/serum digoxin molecules and enzyme conjugates for antibodies coated onto the tube side wall. After aspirating off the mixture, a substrate solution is introduced and incubated in the presence of bound, active enzyme. Substrate conversion is monitored by absorbance change at 405 nm. The method was compared to various RIA assays, and was advertised to have long shelflife and high stability.

XI. FLUORESCENT IMMUNOASSAY: AMERIFLUOR™

Amerifluor™ by American Diagnostics is a heterogenous immunoassay, based on the competitive binding of patient drugs, such as primidone, and fluorescein-labeled primidone with antiserum. A second antibody is added later to precipitate the bound fraction. After separation, the fluorescence of the supernatant is measured, and is proportional to primidone concentration in patient serum/plasma. The technique has been newly introduced, and thus comparison data are not yet available. TSM assays include theophylline, phenobarbital,

phenytoin, carbamazepine, primidone, lidocaine, gentamicin, tobramycin, amikacin, vancomycin, and kanamycin.

XII. PROSTHETIC GROUP LABEL IMMUNOASSAY (PGLIA)

Lin and Pardue recently described a multipoint kinetic method for the measurement of theophylline with homogeneous PGLIA [72]. Drug molecules such as theophylline of patient serum/plasma, and the label (i.e., the drug bound to prosthetic groups of an enzyme, such as theophylline linked to FAD of an enzyme) compete for antibody binding sites. Depending on the patient serum drug concentration, the remaining free prosthetic groups then bind to an apoenzyme, such as apoglucose oxidase. The activity of the resultant holoenzyme, glucose oxidase, is monitored by absorbance change at 520 nm. The method is new and awaits commercial production and further evaluation.

XIII. AUTOMATED TURBIDIMETRIC INHIBITION ASSAY FOR THEOPHYLLINE

This assay, newly introduced by Dupont, is performed by the Automatic Clinical Analyzer. The principle is based upon the competitive binding of theophylline particle reagent and patient theophylline with monoclonal antibodies [73]. Consequently, the extent of aggregation, as measured at 340 nm, is governed by patient drug inhibition of aggregate formation. Because of the binding specificity of the monoclonal antibody, the interference from caffeine and other xanthines is minimized. Figure 21 shows the competitive binding and the reagent for

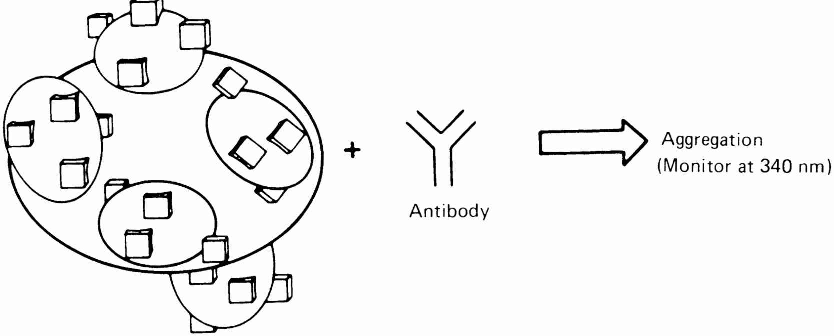


Figure 21 Theophylline particle enhanced turbidimetric inhibition assay. Free drug inhibits aggregate formation. (From Ref. 73.) (Courtesy of Dr. Hamill of Dupont Instrument.)

Table 1 TDM Methodologies According to 1983 Survey Data of AACC (n = 538) and CAP (n = 1095)

	LC	GLC	EMIT[d]	FPIA[e]	TDA[f]	ICS[g]	RIA
Antiarrhythmics							
Digoxin			x	x			x
Dispyramide	x	x	x				
Lidocaine	x	x	x	x			
Procainamide/NAPA	x	x	x				
Quinidine	x	x	x		x		
Antiasthmatics							
Theophylline	x		x	x	x	x	x
Antidepressants							
Amitriptyline/nortriptyline	x	x					
Imipramine/desipramine	x	x					
Lithium[a]							
Antiepileptics							
Carbamazepine	x	x	x	x	x	x	
Ethosuximide	x	x	x				
Phenobarbital	x	x	x	x		x	x
Phenytoin	x	x	x	x	x	x	x
Primidone	x	x	x	x	x	x	x
Valproic acid		x	x	x			
Antihypertensive							
Propranolol	x	x					
Antimicrobials							
Amikacin[b]				x			x
Chloramphenicol[b]	x						
Gentamicin[b]			x	x	x		x
Tobramycin[b]			x	x	x		x
Others							
Acetaminophen	x	x	x				
Acetylsalicylic acid[c]							
Methotrexate			x				x

[a]AA or flame emission photometer
[b]Microbiological assays available
[c]UV/VIS spectrophotometry
[d]Enzyme-multiplied immunoassay testing
[e]Fluorescence polarization immunoassay
[f]Enzyme fluorescence immunoassay
[g]Nephelometric immunoassay

theophylline. The technique is claimed to correlate with EMIT. The application of monoclonal antibody represents one of the first such attempts for TDM. As such, it may represent a potentially important immunoassay development if the technology offers the specificity as advertised.

XIV. CONCLUSION

As a result of the demand for TDM and toxicology measurement, a wide range of methods are now available, as shown by Table 1, summarizing the TDM methodologies according to recent AACC and CAP surveys. Immunoassays, such as EMIT and FPIA, have become the dominant and choice methods, as reflected by the survey results, primarily due to ease, efficiency, and precision. However, a few interference problems exist for some immunoassays for certain patient groups such as renal or pediatric patients [63], and this problem may be minimized by using more specific or monoclonal antibodies. The cost is always higher than that of the chromatographic procedures, which are usually slower and labor intensive. Dedicated analyzers such as QA1 would certainly enhance LC TDM measurements. Presently, chromatography offers possible simultaneous multidrug (total or free) measurements. Interference from other drugs or metabolites may be totally eliminated by appropriate selection of separation parameters. Even though LC has surpassed GC in this subspecialty, GC will always provide unique measurement capability, such as drug screens and GC-MS drug identification. With the increasing popularity of capillary GC, the role of GC awaits further redefinition. The advent of microbore and supercritical LC and their application in TDM toxicology await further studies. Thus, in the eighties, the TDM/toxicology community is indeed entering an exciting era with wide selection of complementary methodologies.

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4

SAMPLING TECHNIQUE

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I. INTRODUCTION

Assurance of the validity of results obtained in an analytical determination depends on the elimination of errors introduced during specimen collection and in subsequent manipulations prior to the final analytical step. As such, variables that affect specimen collection need to be understood and strictly controlled. With that objective in mind, we will review, in this chapter, devices available for specimen collection, the components of materials used in specimen collection devices, and finally, the influence of specimen collection in toxicological analysis illustrated by specific examples.

II. DEVICES

A. Syringes

Prior to the introduction of evacuated blood collection tubes, blood collection was commonly performed using syringes. Both glass and plastic syringes have been used for specimen collection. The permeability of plastics is a limitation for the collection of blood specimens containing volatile drugs. The possibility of plastic components leaching out and contaminating the blood specimen is also of concern. Additionally, adsorption to drugs by plastic surfaces could also be a problem. The subject of permeation, leaching, and sorption from plastic surfaces, in general, has been reviewed [1,2]. Siliconizing in glass syringes can minimize the impact of blood striking the surface and

also minimize contact activation. However, the silicone used for coating the glass interior should be free from leachable contaminants that could affect the assay of drugs. The use of sterile glass or plastic syringes precludes bacterial contamination that would otherwise interfere in the assay of a drug.

B. Evacuated Tubes

These tubes permit collection of a definite volume of blood specimen, which is dictated by the vacuum within the tube. For drug assays requiring serum, blood is collected in a plain, evacuated blood collection tube. In recent years, evacuated blood collection tubes incorporating an inert polymeric barrier material that has a density intermediate between the cellular elements and serum have found wide application. Upon centrifugation, the polymeric material in such tubes rises to seek and form a barrier between cells and serum. Since the barrier effectively screens off serum from cellular material, serum specimens can be stored on the polymeric gel barrier until analysis. Where plasma is the desired specimen for assay of drugs, blood specimens can be drawn in evacuated tubes containing anticoagulants. Just as with plastic surfaces, it is imperative that no leaching or sorptive effects are introduced from the components of evacuated blood collection tubes, such as glass, rubber stoppers, lubricants (such as silicone used to coat stoppers and interior of glass surface), and barrier materials used to aid serum separation, and anticoagulants. The use of sterile evacuated blood collection tubes ensures avoidance of possible bacterial contamination.

C. Needles

The component that first comes into contact with blood during specimen collection is the needle inserted into the vein of the blood donor. Needles used with syringes and placed in holders used for collection with evacuated blood collection tubes are available in a variety of sizes. The gauge number of the needle is indicative of its size. The gauge number is inversely related to the size of the needle. In practice, needles with a gauge ranging from 18 to 22 are widely used. For the collection of multiple blood collection tubes, the multiple-sample needle with a rubber sleeve to eliminate dripping of blood during removal of individual tubes from holder has been the needle of choice. The concern for sterility of such needles does not arise since needles used in blood collection are sterile. Ideally, the lubricant used with needles and associated components should not contaminate the blood specimen, and thus render the results obtained on assay of drugs questionable.

D. Microcollection Devices

Blood specimens from infants, geriatric patients, or patients with veins not amenable to facile venipuncture can be collected in capillary tubes with or without anticoagulant. Devices are available for transferring blood collected in a capillary tube to a plastic tube [3]. The plastic tube may be equipped with an inert polymeric barrier material as an aid to serum separation. Contamination, if any, introduced by such devices should also be addressed.

E. Associated Components

Alcohol swabs used for wiping the surface of the arm prior to venipuncture could be a source of contamination if the alcohol is not thoroughly dried prior to performing venipuncture.

III. COMPONENTS OF MATERIALS USED IN SPECIMEN COLLECTION DEVICES

A. Syringes

1. Glass

The composition of glass used in syringes is either of the borosilicate (type I) or sodalime (type III) variety. The characteristics of such glasses have been prescribed by the United States Pharmacopeia [4]. For short-term contact (which the blood is normally exposed to in the syringe), sodalime glass is adequate even though it has a higher content of alkali then borosilicate.

2. Plastics

Syringes used for blood collection usually belong to the polyolefinic class of resins such as polypropylene or polyethylene. Additives and modifiers are commonly used for these plastics, not only to meet the required physical properties, but also for facilitating the processing of the plastics as well. Antioxidants, antistatic agents, heat stabilizers, UV stabilizers, lubricants, and plasticizers are some examples. These additives and modifiers, if leached out by blood, could interfere in the assay of some drugs.

The type of stopper used on plungers of plastic syringes could also be a limitation. Plasticizers such as di(2-ethylhexyl) phthalate, present in some stoppers, exhibit solubility in lipid vehicles such as blood, and this might interfere in the assay of a drug [5].

Chemical additives like 2-mercaptobenzothiazole, when present as a component of syringe stoppers, undergo transformation upon sterilization to 2-(2-hydroxyethylmercapto)benzothiazole, which has been reported to interfere in toxicological analysis [6].

Stoppers utilizing a plasticizer such as tric(2-butoxyethyl)phosphate (TBEP) are known to displace basic drugs bound to α-1 acid glycoprotein present in serum with the redistribution of the lipophilic drug in the cellular fraction, thus yielding apparently lower drug levels [7]. The effect of TBEP will be discussed at length in a later section of this chapter.

2. *Needles*

The composition of lubricant on the needle may have a bearing on the results of a drug assay. A variety of silicone lubricants have been used for coating needles. A medical-grade silicone suitable for siliconization of materials used in medical devices has found wide application as a lubricant for needles. Interaction of silicone lubricant on needles with blood could pose a problem in terms of displacement of selected drugs from protein binding sites.

Considerations addressed earlier on the subject of the composition of plastics used in syringes also apply to the plastic shield and hub, usually of the polyolefins, used on needles.

The metal content of needles could be an interference when extracted into the blood specimen, since it could interfere in extraction and subsequent chemical reactions that need to be effected in quantifying a particular drug.

3. *Evacuated Tubes*

The composition of glass used in evacuated blood collection tubes is either sodalime (type III) or borosilicate (type I). Containers with sodalime glass are widely used and are adequate for blood collection. The interior of the glass may or may not be siliconized. The type of silicone used may have a bearing in terms of displacement from protein binding sites or evidencing sorptive effects.

The stoppers used with evacuated blood collection tubes are either of the butyl or the nitrile (NBR) variety. The type of plasticizers and additives used to give moldability and the required physical characteristics of stoppers is an important consideration. The classical example of TBEP, which was a component of stoppers used with evacuated blood collection tubes, displacing basic drugs from its binding protein, has been the subject of several papers [8-17]. and has prompted the development of stopper materials free from this plasticizer.

A variety of anticoagulants, such as EDTA, citrate, oxalate, and heparin, have been used in evacuated blood collection tubes. The NCCLS Standard on evacuated blood collection tubes addresses specifications for tubes with and without anticoagulants [18]. Of the various anticoagulants, EDTA, by its ability to chelate divalent cations and by preventing volume shifts between cellular and plasma com-

partments, is claimed to have a stabilizing influence on some drugs, and as such, EDTA plasma is preferred for the assay of basic drugs such as tricyclic antidepressants [19,20].

IV. INFLUENCE OF SPECIMEN COLLECTION IN TOXICOLOGY

A. Methodology

Interferences introduced during specimen collection have been addressed earlier in this chapter. Some of the well-known interferences are the effect of plasticizers on the assay of basic drugs. The effect of displacement of a drug from protein binding sites by a plasticizer such as tris(2-butoxyethyl)phosphate (TBEP) will also be reflected in the analytical method chosen for the assay.

The sensitivity of the analytical procedure to the interfering constituent also has an effect on the assay of a particular drug. For example, the introduction of nitrogen-phosphorus detectors in gas chromatography increased the sensitivity of drugs containing a nitrogen function. It also, however, increased the sensitivity of interfering constituents such as TBEP, and in instances where the drug had the same retention time as TBEP, the drug peak was obscured by TBEP. The binding effects of TBEP will manifest in gas chromatographic procedures by the apparent reduction of peak area if the basic drugs have retention times different from TBEP.

Indeed, prior to the introduction of nitrogen-phosphorus detectors, the conventional flame ionization detector (FID) was unable to detect the interference constituents.

The binding effects of TBEP will also be manifest in high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), and enzyme immunoassay (EIA) procedures. With the variety of detectors used in HPLC, such as electrochemical detection, ultraviolet, and fluorescent detection, the interfering constituent may or may not be detectable.

Immunoassay procedures such as radioimmunoassay depend on the competition of the labeled drug of interest with the drug in serum for binding sites on the antibody. Any interfering constituent that disturbs the competitive effect could introduce an error in the assayed value of the drug.

In contrast, in enzyme immunoassay procedures, the interfering constituent can inhibit or activate the enzyme label and thus affect the amount of substrate transformed to product, thereby affecting the concentration of drug that is measured.

B. Types of Drugs Interfered With

Drugs can be broadly classified as acidic, basic, or neutral. These drugs are transported in the bloodstream bound to a carrier protein. In general, basic drugs are bound to the globulin fraction of plasma, primarily to α-1 acid glycoprotein, and the acidic drugs are bound to albumin. The degree of binding and the stability of the binding protein in vitro influence the analytical result, since the extent of redistribution of free drug into the cellular fraction will depend on its affinity of binding to its carrier protein and the stability of this protein. In general, basic drugs are highly protein-bound; thus displacement of such drugs from these binding sites by an interfering constituent will have a pronounced influence on the analytical result. It is not surprising then that the widely reported interference of the plasticizer TBEP has been restricted to basic drugs, such as quinidine [11], propranolol [8], lidocaine [12], tricyclic antidepressants [10], alprenolol [13], meperidine [14], perphenazine [16], chlorpromazine [15], fluphenazine [16], and trifluoperazine [16].

C. Types of Interfering Substances

In addition to the well-known effect of organic phosphates such as TBEP, other sources of potential interferences should not be overlooked. Phthalates, such as diethylphthalate have contributed overlapping peaks in gas chromatography, thus, increasing the peak area of a drug having a retention time similar to that of the phthalate compound. Sorptive or displacement of drugs from protein binding sites could occur with some types of silicone used to coat the interior of a glass tube. Evacuated tubes containing gel separation devices to effect separation of cells from serum upon centrifugation should be validated by comparing them to an accepted blood collection system to insure that there is no loss of recovery of the drug due to adsorption to gel barrier material and that no displacement of drug from protein binding sites occurs.

D. Quality Control

It is difficult to predict for each drug and methodology what types of analytical interference can be encountered. As a quality control procedure, whenever a new type of collection system or a new lot of evacuated blood collection tubes is used, the tubes should be evaluated for potential interference. One recommended scheme [21] is to draw three blood samples from a subject at an appropriate time subsequent to the administration of a drug, using a glass syringe needle system (plastic syringes can be evaluated in the same manner as a total system); one evacuated blood collection tube held in a vertical position subsequent to blood collection insuring minimal contact of blood

with the stopper, and another evacuated blood collection tube inverted approximately ten times subsequent to blood collection to insure thorough content of blood in the stopper, and storing that tube in a horizontal position to allow intimate contact of blood with both glass and stopper.

E. Relative Merits of Anticoagulants

When plasma is desired and when whole blood is needed as well to study the whole blood-to-plasma distribution ratio, a choice of anticoagulants must be made. Both EDTA (ethylenediaminetetraacetic acid) and heparin have been used as anticoagulants to collect specimens intended for drug analysis [22.23].

Although both heparin and EDTA are suitable for purposes of obtaining whole blood, EDTA is believed to be the optimal anticoagulant and the specimen of choice for measuring levels of tricyclic antidepressants since EDTA, by chelating divalent cations, might contribute to the stability of these drugs by protecting them from oxidation.

F. Current Knowledge

As awareness of interfering constituents introduced by specimen collection in the assay of drugs increases, attempts will be made to overcome these interferences, thus minimizing interfering effects of specimen collection. The focus on the effects of the plasticizer TBEP in the rubber formulation of certain brands of evacuated blood collection tubes has served as an impetus for elimination of this contaminant. The elimination of this plasticizer from the rubber formulation of evacuated blood collection tubes (VACUTAINER brand) has rendered it suitable for the assay of drugs interfered with previously. Thus, tricyclic antidepressants were successfully assayed with an HPLC procedure [20,24]. Quinidine, procainamide, and *N*-acetylprocainamide were assayed without any interference by a gas chromatographic procedure utilizing a nitrogen/phosphorus-selective detector [25]. The TBEP-free evacuated blood collection tubes have been used successfully with enzyme immunoassay and radioimmunoassay procedures. In addition to the drugs mentioned above, drugs such as propranolol [26], haloperidol [23,27], thioridazine [28], and mexiletine [29] have been successfully assayed with specimens collected in evacuated blood collection tubes free of TBEP in the stopper formulation.

V. CONCLUSION

With the development of ultrafiltration membrane filters for separating free drug from protein-bound drug, and the development of HPLC procedures for measuring both the parent drug and its active metabolites,

quality control of specimen collection becomes of paramount importance.

With new drugs introduced every year together with even newer analytical procedures, hitherto unknown interferences introduced by specimen collection are bound to be uncovered. The optimum specimen collection device should be free from any such potential interference. This, it is to be hoped, can be achieved by quality control of specimen collection devices, and validation of such devices in the clinical laboratory.

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5

COMPUTER CONTROL OF LIQUID CHROMATOGRAPHIC ANALYSES

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I. INTRODUCTION

The advent of compact, inexpensive computers has made the automatic control of analytical apparatus readily achievable, and the data gathering from such apparatus reasonably straightforward. The analytical usefulness of a liquid chromatographic separation is critically dependent on a number of variables, principally the polarity of the mobile phase and the gradient of that polarity in nonisocratic operation. Moreover, the constancy of the flow-rate and, to a lesser extent, the stability of the operating temperature are important for reproducible results. The precision of a quantitative liquid chromatographic analysis is also dependent on the integrity of the injection system, both from the point of view of sample size, and also from the point of view of the injection concentration profile. The importance of a very narrow injected band of solutes has been heightened by the extreme efficiencies obtained using microparticle adsorbents as stationary phases.

From these considerations alone, the automatic operation of a chromatograph is desirable, eliminating operator error to a large degree, giving greater precision to the control of the separation variables and allowing greater flexibility in the controlled variation of parameters during the development of the chromatogram. However, the value of computer-controlled liquid chromatography lies chiefly in the data gathering capability and the capacity to process data immediately, to store them, and to express them in a prescribed format. The signal from a number of detectors can be digitized, peak areas obtained using more or less simple algorithms, and absolute results obtained from internal calibration with no reference from the

operator beyond sample preparation and loading. With the appropriate magazine for samples, the instruments can be run 24 hr a day, and several such systems maintained and overseen by a single operator. For strictly routine separations and analyses, this arrangement is clearly ideal although automatic control and data gathering has a major disadvantage which must not be overlooked. This disadvantage lies in the distancing of the operator from the chromatography by the computing instrumentation. It is now possible to run a series of analyses on a computer-controlled instrument without the least idea what the chromatogram looks like. The instrument carries out peak identification according to a program entered perhaps months before, and performs an integration on partially resolved peaks according to an algorithm that makes certain assumptions which are all too readily overlooked by the operator. Since under almost all conditions, if samples go in one end, numbers will inevitably come out of the other, there is a tendency to place unwarranted trust in the analytical system. This is exacerbated by the fact that the simplicity of some automated instruments encourages the use of personnel with only a very limited training and correspondingly little understanding of the processes undertaken by the computer and the limitations from which it suffers.

The major purpose of this chapter, therefore, is to describe the various aspects of computer control of a liquid chromatograph commonly employed for analysis of drugs and their metabolites, in such a way as to bridge the gap between the chromatographic processes and the computed data presented to the operator. A description will be given, therefore, of the liquid chromatographic system and the means by which a computer is enabled to control it and to gather data from it, and the automation of liquid chromatography systematized to allow the potential purchaser to decide on the kind of system suitable for his or her needs. While no attempt will be made to give a complete review of the different instruments commercially available (such a review has recently appeared [1]), reference will be made to various commercially available instruments, and the construction of a simple computer-controlled liquid chromatograph from individual units linked with suitable interfaces will also be considered. The aim will be to provide a sufficient outline of the technical aspects of computer-controlled liquid chromatography so that the user might identify the advantages and limitations of any such system operated.

II. THE ANATOMY OF A COMPUTER-CONTROLLED LIQUID CHROMATOGRAPHIC SYSTEM

A. Principal Components

Figure 1 shows a sketch of the principal components of a liquid chromatographic system designed for general-purpose analytical applica-

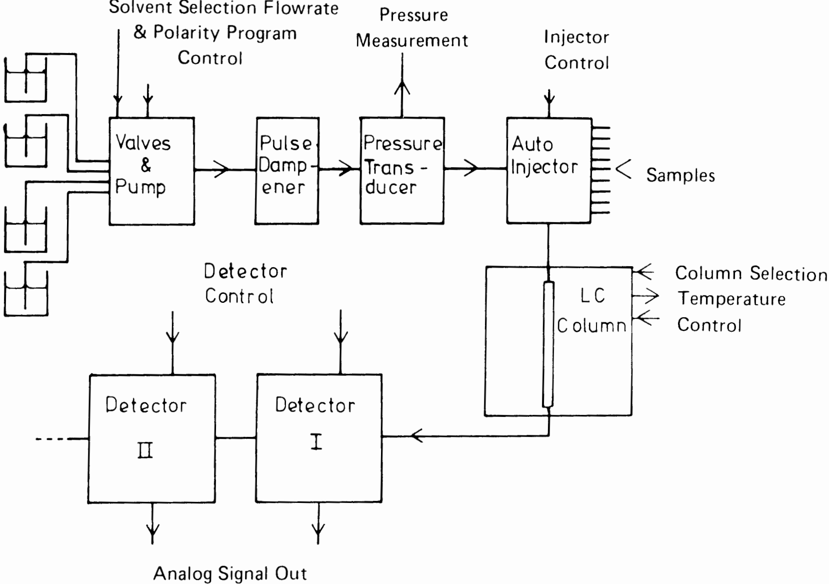

Figure 1 Block diagram of automated liquid chromatographic system showing points where computer control can be introduced.

tions. The arrows show the points in the system where computer control and measurement can be utilized, and are shown in two directions: those pointing towards the system indicate that the computer is giving instructions to that part of the system, while an arrow pointing away indicates that information is being derived from that section and fed to the computer. In some cases a pair of arrows is used indicating that data are passing both ways, placing the item in the system under the most direct and positive control of the computer. Each point of control requires an interface device and a transducer. In the case of arrows pointing towards the chromatographic system (*motor* control, using biological terms), the interface device must translate a digital signal (either a static content of a data register or a stream of binary digits) into a signal suitable for the control of the particular system variable. This signal is sent to the transducer which actually effects the control. Thus in the case of the the flow-rate control, the desired flow-rate is fed as binary digits to the interface unit which then produces an analog signal which is used to govern the speed of the pump which is in this case the transducer. In the case of *sensory* functions (arrows pointing away from the system), a transducer is used to develop an analog signal which is then digitized using an appropriate interface and the digital signal accessed by the computer. The mea-

surement of temperature is a good example: the thermocouple, or thermistor is the transducer, developing a signal fed to an analog-to-digital (A/D) convertor as an interface device. If the two signal directions are combined, the computer is enabled to exert complete control over the variable, of whatever kind is suitable: proportional, derivative, or integral, with the parameters governing the control set within the program. In this way, the control of the variable is exceptionally fine and its programming with time, according to any preselected function, can be readily achieved. The block diagrams in Figure 2 show the components for both *motor* and *sensory* communication between the computer and the liquid chromatographic system.

B. Flow-rate and Gradient Elution Program Control

Most commercially available liquid chromatographs are fitted with twin piston reciprocating pumps, the strokes of which are exactly out of phase to enable a constant, pulseless flow to be achieved. Under isocratic conditions, the strokes of the pumps are fixed (usually the total displacement per stroke is 0.1 ml approximately) and the speed at which the pistons reciprocate is used to vary the flow-rate. This is carried out by a stepping motor driven by a stream of pulses from the computer, the frequency of such pulses being controlled by the contents of a register which forms part of the computer working memory. In most cases two such dual position pumps are provided, each driven by its own interface and each supplied with clock pulses which determine the rate of rotation of the motors. When a particular flow-rate is selected, the frequency of the pulse stream is fixed at the value which corresponds to that flow-rate. Arranging the contents of the register which governs this clock frequency to vary with time enables the flow-rate to be programmed according to any predetermined function.

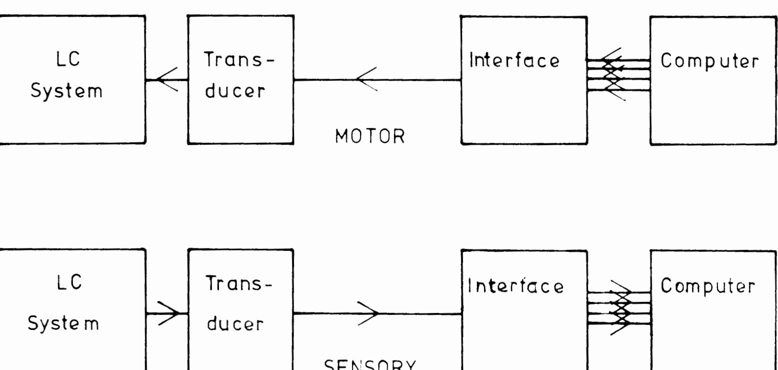

Figure 2 Block diagram showing elements required for *motor* or *sensory* communication between liquid chromatograph and computer.

Pressure sensors are provided which enable the computer to determine the working head pressure and operate fail-safe routines should the pressure exceed a preset level (risking damage to gauges and injection systems, etc.) or fall below a lower limit (as resulting from a potentially hazardous leak).

Gradient elution is achieved by one of two methods: In the so-called dual-pump mode, two pumps each with two cylinders of the type described above are fed with the two solvents used to obtain the polarity gradient. The computer controlling these pumps causes them to deliver the flow appropriate to achieve the mixture prescribed by the polarity program specified by the user. These flows are mixed on the high-pressure side of the pumps and fed to the injection system. In the single-pump method, mixing occurs on the low-pressure side of the pump by means of a solenoid valve which selects the composition of mobile phase required by continuously switching between the two solvent reservoirs with a mark-to-space ratio equal to the desired volume composition of the mobile phase. This valve is under control of the computer (or of a slave microprocessor), and so the polarity gradient is infinitely variable according to the function programmed into the computer. Some systems have a refinement in which the valve is shut off completely during the delivery stroke of the pump which improves the precision of the gradient profile.

The main advantage of dual-pump operation is that the gradient is somewhat more precise than that produced by the single-pump system. Also, since solvent mixing occurs on the high-pressure side, observation indicates that solvent degassing is less critical. The disadvantage is that the pumping system is virtually twice as expensive. From the point of view of computing, there is little to be said in favor of one system over the other, except that, in the case of multisolvent programming, the addition of a multiway valve to the single-pump requires only modest modification to the control software.

If precise, repetitive analyses involving gradient elution are to be carried out, a dual-pump system is to be preferred, and this arrangement is becoming progressively standardized in commercially available instruments. While a dual-pump system is capable of infinitely variable solvent delivery, in practice, many manufacturers limit the flow and polarity programs which are possible with a particular instrument by building into the control computer software a predetermined mathematical function into which parameters are inserted to give the user control over the general shape of the profiles. For flow programs, the computer usually divides the separation time into a number of periods (usually up to 10) in which the user can specify flow-rates and the gradient of the desired flow-rate change in the period. For polarity programs using two solvents, a function is often used to allow nonlinear concentration changes with time. A commonly used function of this type is:

$$Q(t) = Q(i) + [Q(f) - Q(i)] \left(\frac{t}{T}\right)^n \tag{1}$$

where Q(t), Q(f), and Q(i) are the compositions of the mobile phase (expressed as a volume fraction of one component) at times during the development of the chromatogram, at the end, and at the beginning of the development, respectively; T is the total separation time, and n is the parameter whereby the function shape is varied. Figure 3 shows the polarity profiles which are available using this function.

This method of programming is very convenient for most liquid chromatographic applications since it allows wide flexibility in polarity programming while retaining simplicity in the specification of the program required. The operator has to insert desired values for T, Q(f), Q(i), and n, only. However, this method does not give complete control to the operator and it is conceivable that if the very best separation of a multicomponent mixture were required, a more versatile programming facility might be necessary. In this case the operator would require an automated liquid chromatograph which gave access to the registers within the computer which prescribes the rate at which the pumps deliver solvent. The user could then write a control program which would cause the pumps to deliver solvent according to any function desired. The most convenient arrangement for this is provided by a pump unit driven from a computer which has a

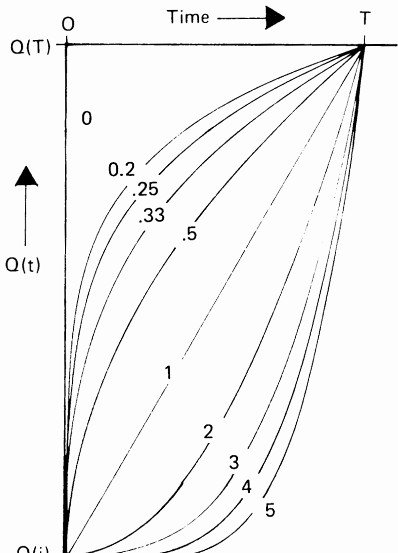

Figure 3 Gradient elution concentration profiles for binary solvent systems using different values of n in Eq. (1).

BASIC interpreter installed. Alternatively, the pump unit used should have a suitable interface port (RS232 or similar) to allow control by an external computer.

C. Automatic Injection Control

A computer-controlled liquid chromatograph can be fully exploited only if automatic injection systems are employed, enabling the instrument to run over long periods virtually unattended. Essentially, the automatic injector in common use consists of a mechanical arrangement by which samples are transferred from a magazine of vials to the injection head of the chromatograph by means of a mechanically operated syringe or by a mechanically operated loop filling system. In order to be truly flexible, such a system must have the capacity of a large number of variable parameters. These include the frequency of injections, the injection size, syringe washing routines, the number of injections per sample vial, etc. The function of the computer or control microprocessor is largely that of a versatile and complex sequence timer, although more complex decision-making is also possible. For example, it is a simple matter to arrange for a computer-controlled chromatograph to run a single chromatogram repeatedly until statistical analysis of the results shows that a specified confidence limit has been reached. Equally, it is readily possible to run the same sample under a wide variety of mobile-phase compositions and gradients to enable a separation to be optimized virtually automatically.

Of the two commonly employed injection systems, syringe or loop fill, the former has the disadvantages normally associated with the manual use of syringes, namely, only moderate reproducibility of injection volume and some liability towards failing to fill adequately, but in general, the automatic use of syringes gives more relaible performances than manual use. It is necessary for the automatic injection system to have the facility for immersing the needle in solvent when it is not in use or blockage can occur. In the loop-fill injection method, the septum cap of the sample vial is pierced by two needles, and a small pressure of nitrogen applied through one such needle drives the sample into the other and thus into the loop. The precision of the injection volume obtained in this way is very high.

The automatic injection system using a syringe or loop is mechanically complex if a large number of different samples have to be transferred from vials to the column head, and it is essential that the system shows high reliability since it will run for long periods of time unattended. The interface required for the operation of such a system is relatively simple, however, since the mechanical movements required to accomplish the injection are all readily achieved using solenoid valves. Figure 4 shows a simple interface for the operation of three valves from the user port of a Commodore 3032 computer. Table 1 shows a short segment from a BASIC program by which the relays from

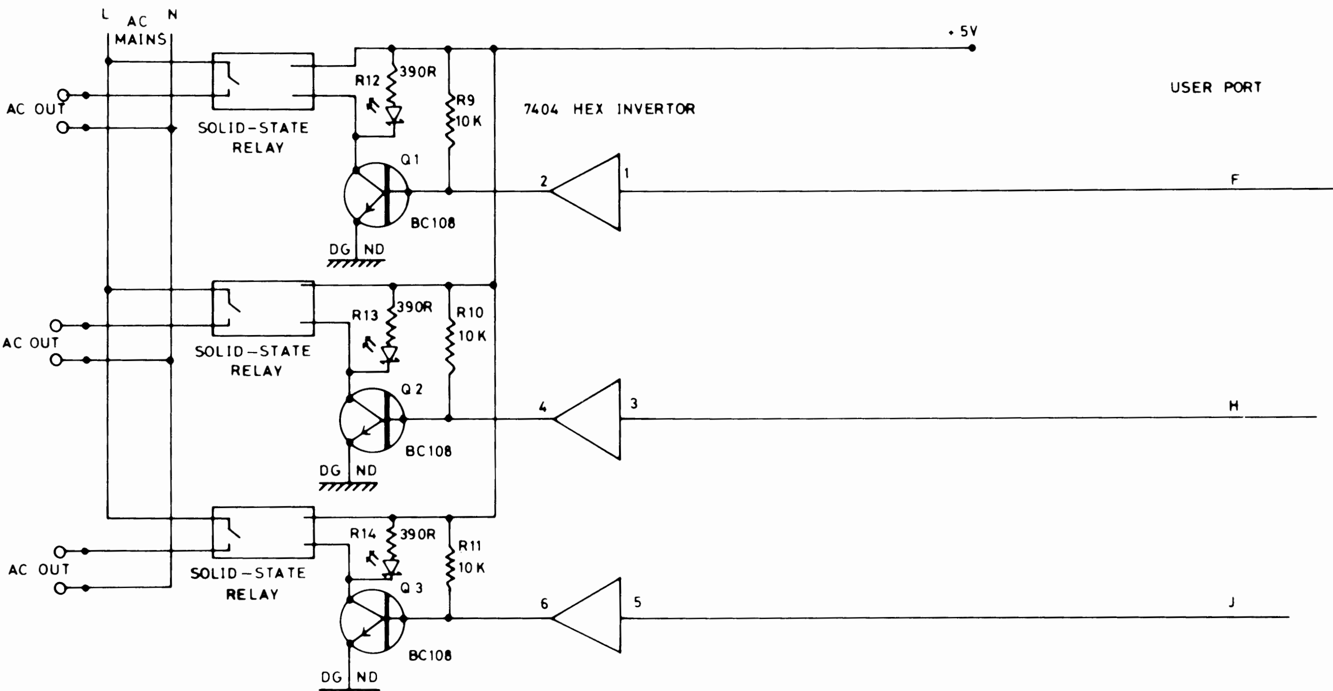

Figure 4 Circuit of 3-channel interface for the control of solenoid valves from a Commodore 3032 computer. (Reproduced by permission of Preston Publications, Inc.)

Table 1 BASIC Program Statements for the Control of AC Mains Outlets

BASIC statement	Event
300 Z = 59471	
310 POKE 59459,56	Places 56 in data direction register, making pins F, H, and J on user port all outputs.
320 POKE Z, 8 330 POKE Z, 32 340 POKE Z, 32	Examples of statements which alter the logic level of pins F, H, and J on user port.
350 T = TI	Introduces a time delay of 1 sec into program execution.
360 IF TI < (T + 1*60) GO TO 360	Lines 320-340 and timing lines 350-360 can be arranged to produce any pattern of events desired.

Fig. 4 can be controlled. Further details are given in Refs. 2,3. The logic levels placed in three bits of the parallel user port register are fed via the inverters to the bases of switching transistors which control commercially available solid-state relays. These in turn provide switched AC mains outputs with which to drive appropriate solenoid valves. If only three valves are required, and the user port is not in demand for other purposes, the independent control of each relay by a single bit as shown is satisfactory, but if the control of more valves is required, it is advisable to insert a 3-bit binary decoder between the port and the interface so that the total number of valves operated from n bits becomes $2n - 1$.

The sequencing of such a valve arrangement is readily carried out by means of a BASIC program, although most commercially available automatic injection systems are provided with a more restricted range of options which require less knowledge of computer programming to implement. For almost all analytical purposes, however, the restricted range of control given to the operator is sufficient and the commercial instruments entirely satisfactory. If specialized sampling is required, for example, from a series of chemical feedstock streams, then valves controlled from a microcomputer by an interface as shown in Fig. 4 are, however, more suitable.

D. Column Selection and Oven Control

If a liquid chromatograph is to be used for a wide variety of routine analytical separations, it is most convenient if the selection of the appropriate column can be carried out automatically. A chromatograph could in this case be fitted with several columns, and the computer would select the one required at the same time as it selects the vial from which the sample is withdrawn. More complex multiple column arrangements are also possible: a sample may require that after a preliminary separation on a first column, part of the eluate should be fed to a second column and part to a third, to achieve a complete analysis of a complex mixture. This kind of analysis required that a valve be operated at the correct point during the elution of the chromatogram from the first column, and this can be readily carried out with precision by means of a computer and an appropriate interface and solenoid valves of the type shown in Fig. 4. If the use of multiple columns is envisaged, the most important property of the computer-controlled liquid chromatograph is that it be sufficiently flexible to enable the control of several valves from a simple program. The user needs to be able to construct his or her own system and place it under the control of the computer. The more sophisticated commercial instrument is thus not always appropriate for a limited specialized task, as it is frequently more difficult to modify than to construct a suitable system from separately available units.

The control of column ovens is also a task suitable for the control computer. As indicated above, it is possible to arrange for the computer to measure the temperature and control the heater directly. This method, although flexible and precise, places a further continuous background job in the computer, and it is probably better for the computer to send a signal to a proportional controller, setting the temperature. In this arrangement, the job of controlling the temperature is carried out by a conventional analog proportional controller with the temperature set by the signal from the computer. If the temperature is required to be measured by the computer, a further sensor and interface can be provided.

E. Detector Selection and Control

The eluent from the liquid chromatographic column enters either a single detector or passes through a series of detectors, the choice of which can be controlled by means of a computer as can the operating parameters of the selected device. It is usually preferable to avoid the use of valves or any unnecessary pipe work between the column outlet and the detector, and for this reason the most convenient arrangement, when more than one detector is used, is a simple series of configurations, as indicated in Fig. 1. Data can be sampled into the computer

via analog-to-digital convertors attached to the electrical signal output of each detector.

Circumstances do arise when the parameters of the detector itself have to be under computer control, and this is particularly the case when a variable-wavelength, UV absorption detector is used. The wavelength used is selected by a monochromator controlled by a stepping motor driven from a series of pulses generated from the controlling microprocessor. This is a refinement which is necessary only when separations have to be carried out with different UV absorption wavelengths in the course of a series of unattended analyses.

III. COMPUTER-CONTROLLED COLLECTION OF LIQUID CHROMATOGRAPHIC DATA

We can now turn to what is the most valuable attribute of computer-controlled liquid chromatographs, namely, their potential for the automatic data gathering and processing.

A. Basic Principles

In order that analog voltages from detectors, etc., may be measured automatically and be in a form in which they may be manipulated by the computer, an interface device is required between the detector and the computer. A block diagram of such an interface is shown in Fig. 5 which allows a single channel of analog data to be sampled under program control. The input amplifier may be a simple operational amplifier which scales the applied signal to a range appropriate to the A/D converter, or it may be more elaborate, automatically adjusting its scaling factor so that the signal applied to the convertor always remains within a certain range. This elaboration improves the signal resolution at low levels but is more complex since the computer must be

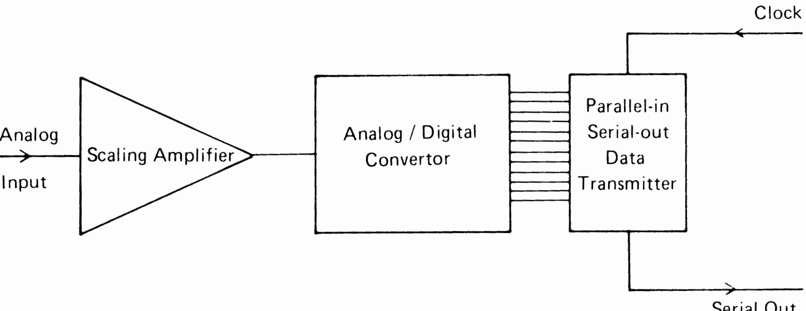

Figure 5 Block diagram showing elements of an interface for computer sampling of an analog voltage signal.

fed with information which identifies the current range of the scaling amplifier. The signal, thus appropriately scaled, is applied to the analog to digital (A/D) convertor which enables the analog voltage signal derived from the detector to be digitized and fed to the computer in a form in which it can be processed. The different types of such convertors available have been reviewed by Reese [4] and essentially fall into three groups: those using a successive approximation method, those using a voltage-to-frequency conversion, and those using a dual-slope integration of the analog signal. The devices of the first type contain a digital-to-analog (D/A) convertor, a counter, a scaling amplifier, and a comparator. A clock signal is provided which is fed to a counter, the output of which is fed to the D/A converter, so that an analog signal is produced which increases progressively with the clock. It is continually compared with the scaled input voltage by means of the comparator, and when the voltage synthesized by the D/A convertor reaches the value of the scaled input voltage, the clock is interrupted, and the current content of the counter is equivalent to the scaled input voltage. The main advantage of this system is speed, but it is very sensitive to high-frequency noise, and is thus only used in chromatographic applications when a very high speed is essential (for example, where a large number of chromatographs are coupled to a single computer) and when adequate precautions are taken against high-frequency interference. It is often found that high-frequency noise from triac power controllers, for example, is difficult to overcome when convertors of this type are used.

The second type of convertor is in itself very simple and consists of a voltage-controlled oscillator, the frequency of the output being proportional to an applied input voltage. A digital measure of this voltage is thus obtained by feeding the output frequency into a counter which is gated with a fixed counting interval. Although this is a simple device it requires a more complex control program, but is fast and reliable, and is used in some commercial instruments.

The third type of convertor, the dual-slope integration method, although more complicated, is widely used in computer-controlled chromatographs, and so its manner of operation deserves some description. The main components of the convertor are shown in Fig. 6. At the start of the conversion, the counter is set by a pulse on the reset line and the switch control bistable allows the signal required to be measured onto the input of the integrator. The counter will be clocked upwards during this integration until the most significant bit goes to logical 1, whereupon the input switch is caused to select the reference voltage, and the integration of this (because it is of the opposite sign to the signal voltage) results in the integrator output falling to zero. When this point is reached, the counting stops and the contents of the counter represent a number proportional to the applied voltage. Thus the first integration accumulates a charge on the integration capacitor proportional to the applied voltage, since the

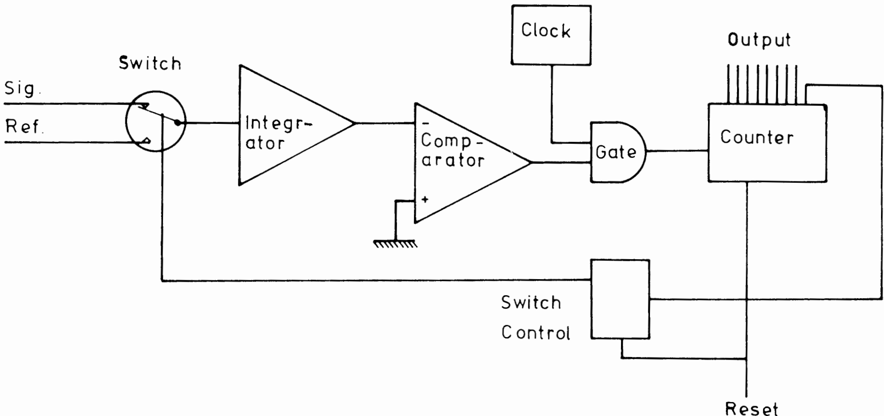

Figure 6 Diagram showing the components of dual-slope analog-to-digital convertor.

time of integration is fixed by the clock speed and the counter capacity. The second, downward integration measures this charge by displacing it by integrating a fixed reference voltage, the time required for this displacement being recorded as the number of pulses accumulated in the counter when the conversion is complete. This time is proportional to the applied signal voltage. The main advantage of this method of A/D conversion is precision, the dual-slope integration method enabling many errors to cancel out, although it is not particularly fast. The conversion time is typically 50-200 msec, but this actually is an advantage in chromatographic work because noise of a higher frequency than the conversion frequency is attenuated.

In most commercial liquid chromatographs, the A/D convertors are those giving at least 12-bit outputs and are a little more complex than indicated in Fig. 6 in being able to digitize voltages of either sign, incorporating a sign detector, the output of which is fed to the computer as a separate bit of data. The output of the counter in Fig. 6 is fed into the computer by means of the data transmitter (Fig. 5). Two main types of these are in use: the series type and the parallel type. In the series type, the data in parallel form are fed into a shift register and then clocked out under program control into a similar shift register within the computer as a stream of pulses. This internal register is capable of being read under program control, and thereby the data enter the computer database field. In parallel transmission, the data are presented to the computer bus via a series of gates so that access may be gained to them when a control program permits. Either way the contents of the A/D counter appear in the computer storage at a specified memory location.

For 16-bit microprocessors, all of the data from a single conversion are transmitted together and the transfer is essentially a single operation, but for 8-bit processors the data transmission is carried out in two stages, 8 bits at a time. This is fractionally slower and requires a more elaborate control program.

B. A Simple Chromatographic Data-Gathering System

While many liquid chromatographers will exploit the computer control systems available commercially, brief space will be given here to the detailed construction of a single-channel interface unit specifically designed for data gathering from a chromatographic detector into a Commodore 3032 computer. The advantage of the simple system presented here is that it is very flexible, being applied to a wide range of chromatographic detectors, and it is inexpensive and couples to a very widely used and readily available computer. It has been previously reported [2], and applied to a specialized series of chromatographic techniques [5], but is presented here as an example of the application of principles outlined above.

The circuit of the analog-to-digital convertor module is shown in Fig. 7. The circuit is based upon the AD7550 large-scale integrated circuit (Analog Devices, Norwood, Massachusetts), which is of the quad-slope integrating type (a development from the dual-slope type described above). The full specifications of the device are available from the manufacturers. The input signal from the detector is applied to the scaling operational amplifier OA1, the gain of which is set at 100. The amplified signal is applied to the analog input of the convertor, and two reference voltages applied from zener diodes. Components R1, C1, and C3 control the time constant of the integration amplifier and the clock frequency, which is set at approximately 250 kHz.

With the analog signal present at the input of the convertor, a conversion is initiated by a pulse from the computer at least 400 nsec wide fed to the START pin (pin 14). Conversion actually starts on the high-to-low transition at the end of this pulse. This start pulse is generated from pin C of the computer user port, this pin being the least significant bit of an 8-bit register extending from pins C to L. Data can pass to or from the computer via this register, the direction of data transfer being controlled by the contents of the data direction register in the computer. The contents of both these registers are controllable from a BASIC program in the machine. During the conversion, pin 36 of the device (BUSY) remains at logical "1" and this signal is fed to the computer port. This allows the computer to detect the end of the conversion process. When this is reached, the digital result of the conversion is present as a 12-bit binary number at the output of the A/D convertor. At the same time, pin 36 falls to logical

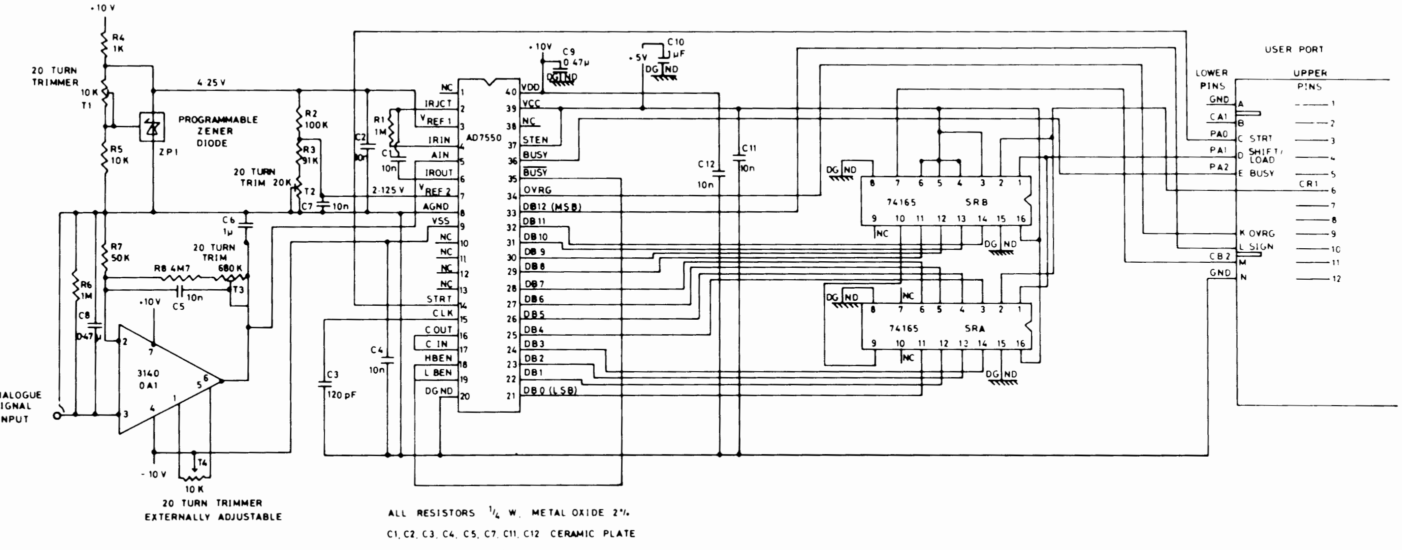

Figure 7 Circuit diagram of a single-channel interface for data gathering from a chromatographic detector by a Commodore 3032 computer. (Reproduced by permission of Preston Publications, Inc.)

Table 2 BASIC Subroutine for Control of A/D Convertor

BASIC statement	Event
100 DIM X (1000), T(1000)	
110 Z = 59471: A = 0	59471 = address of user port register.
120 Z6 = 59466	59466 = address of internal shift register.
130 Z7 = 59467	59467 = address of auxiliary control register.
140 Z9 = 59469	59469 = address of interrupt flag register.
145 POKE 59464, 1	
150 POKE 59459, 3	59459 = address of data direction register. Sets: clock rate for shift pulses for data transfer and places binary equivalent of 3 in data direction register, making pins C and D of user port outputs all other pins inputs.
155 TI$ = "000000"	Zeros clock.
160 FOR J = 1 to 100	Causes the computer to perform 100 calls of the A/D conversion subroutine.
170 GOSUB 1000	
180 NEXT J	
190 STOP	
1000 POKE Z, 1 + A	Sends start pulse to pin 14 of A/D convertor.
1010 POKE Z, 0 + A	

1015 T(J) = TI/60	Stores current time in seconds in array element T(J)
1020 IF (PEEK (Z) AND 4) GO TO 1020	Tests the status of BUSY and waits for conversion to finish.
1030 POKE Z, 2 + A	Places external shift registers in shift mode.
1040 Y = PEEK (Z6)	Resets the internal shift register interrupt flag.
1050 POKE Z7, PEEK (Z7) AND 2770R4	Sets the auxiliary control register such that 8 pulses are output on pin 6 of user port, shifting 8 bits of data into internal shift register, while the program waits for the setting of the shift register
1060 WAIT Z9, 4	interrupt flag, which occurs on completion of data transfer.
1070 X = PEEK (Z6)	Transfers the contents of the internal shift register to variable X and resets the shift register interrupt flag.
1080 WAIT Z9, 4	Waits until a further 8 bits of data have been shifted into the internal shift register.
1090 X = X*256+PEEK (Z6)	Compiles both 8-bit bytes of data into a single integer in variable X.
1100 IF (PEEK (Z) AND 129) GO TO 1130	Tests sign of input.
1110 X(J) = (8192-X)*20/8192	For positive numbers, X is the binary complement of the voltage and is here inverted and scaled
1120 GO TO 1140	0 to 20, for storage in variable array X(J).
1130 X(J) = X*20/8192	If voltage is negative, X represents the noninverted voltage and is merely scaled 0 to 20.
1140 RETURN	

"0," thus indicating that the conversion is complete. Under program control, the 12 bits of data are then loaded into two 8-bit parallel-in, serial-out shift registers SRA and SRB (in Fig. 7). Then, again under program control, the data is shifted into an internal shift register in the computer in two blocks of 8 bits. This is necessary because the computer for which the interface was designed is an 8-bit device, and thus can accommodate the data only in bytes this size. Since the data derived from the A/D convertor was 12 bits in length, this is treated as two 8-bit bytes with 4 leading zeros in the more significant byte. When the data has been shifted, the control program calculates and scales the result and places it in a memory location, after which a further pulse is sent to the START pin and a further conversion is thus initiated. The BASIC program which controls the convertor is shown in Table 2, and further details which allow the construction and implementation of the system are given in Refs. 2,3.

While the system described above is of the type illustrated in Fig. 5, because there is such diversity in the exact specification of computer access ports, it cannot be regarded as a universal A/D convertor interface. Nevertheless, if the specifications of the port of any computer are known, the design of a suitable interface presents little problem. The scaling amplifier circuitry and the convertor arrangement remain unchanged, but the data transmission circuit has to be tailored to the computer employed. If modest electronic constructional facilities are available with some moderate expertise in computing, existing nonautomatic chromatographs can be updated to an automatic data-gathering operation using inexpensive microcomputers, at a cost far below that of a new automatic chromatograph. The other advantage of this approach is that the system can be designed to conform exactly to the analytical requirements, and investment in redundant costly facilities is avoided.

IV. DATA HANDLING

A. Retention Data

By means of the interfaces described in previous sections, the computer can gather a series of numbers which represent the signal level from a detector over a period of time, sampled at a prescribed frequency. In the case where the computer is gathering data during the development of a chromatogram, the numbers so obtained will represent the concentration profile of various eluates as they emerge from the column. The chromatographer will wish to gain a measure of the retention of these eluates, and very often a quantitative determination of their concentrations in the injected sample. In order to carry out the first of these tasks, the computer has to be supplied with a signal at the point of injection, which can be derived manually or, where an automatic injector is used, from the control program itself. For

symmetrical peaks, the retentions are sufficiently characterized by the position of the peak maximum, and the time for this is determined by numerical differentiation of the digitized signal, recording the times at which the sign of the derivative changes from positive to negative. For the calculation of corrected retentions, relative retentions, and Kovats indices, standards need to be run in the same or separate chromatographic analyses. Thus peak identification is significantly assisted by a computer, there being no need for any measurements on chart paper, and since the timing is usually carried out from an internal quartz clock under program control, the results are precise. With an automatic injection system, errors associated with the manual manipulation of syringes are eliminated and the repeatability of chromatograms improved. There is, however, one disadvantage of computer-determined retentions, apparent if a visual display of the chromatogram is not obtained. Simple retention measurements based on a search for peak maxima may ignore peak asymmetry and peak distortion due to partial resolution of the components of a mixture. The chromatogram must always be examined visually; otherwise, results can be very misleading.

The main advantage of computing in carrying out this kind of qualitative chromatographic analysis, apart from ease and precision, is that calculations can be carried out on a chromatogram after it has been completely eluted. It is not necessary to determine the positions of the peak maxima as they occur during the development of the chromatogram. The entire elution profile is simply digitized and then subsequently manipulated under program control to determine the required parameters. This is important if the development of the chromatogram is very rapid or the chromatographic efficiency of the peaks is greater than 50,000 theoretical plates, and the manipulating program is not in machine code. Programs written in BASIC are usually sufficiently slow to make extensive calculations between sampling impossible without reducing the sampling frequency to an unacceptable level. On the other hand, to write the whole program in machine code is often an arduous task. A much better compromise is to write the data-gathering subroutine in machine code and to sample as rapidly as desired, and then, with the chromatogram completely digitized, to perform further calculation in a BASIC program. This applied, of course, only where an automatic chromatograph is set up in the laboratory. Where commercial instruments are used, the programming has already been carried out such that it will run with sufficient speed for normal analytical purposes.

B. Peak Integration

The more useful and by far the most complex operation carried out on the computer, however, is the task of integration of peak areas to obtain quantitative information on the composition of the injected sample.

It happens that the human eye is actually much better than the present computer programs at estimating the appropriate approximations to be made to obtain the integration of a partially resolved peak. It has been found difficult to determine the criteria by which a computer might decide whether the most accurate integration results, say, from dropping a perpendicular from the valley between two such peaks to the baseline, or perhaps drawing a tangent across the base of one of them. This means that even when the chromatographer has at his disposal sophisticated integration software, he is strongly advised to apply his own constraint to the manner in which the integration is carried out, and most commercially available systems do present the operator with some such options.

In very many cases, it is easier to improve the chromatographic separation than to make the integrations more elaborate to deal with partially resolved peaks. In quantitative analysis, partially resolved peaks should by tolerated only where they are absolutely unavoidable and even then, the manner in which the integration is carried out must be scrutinized to be sure that it accords with the chromatographer's own estimate. The integration of a chromatographic peak involves a number of separate operations. These are (a) peak recognition, (b) peak integration, (c) end of peak detection, (d) background evaluation and subtraction from total peak area, and (e) in the case of partially resolved peaks, more elaborate calculations. These have been dealt with in detail [6,7], but the chromatographer needs to be aware of the general criteria by which these various decisions are made by the computer.

The most common method of peak detection is the current slope of the signal exceeding a preset positive value for a number of consecutive measurements. This arrangement inhibits false peak detection by rapid spikes but allows positive identification of even small peaks. The author has found that the threshold value for this test should be set between 0.01 and 0.1 mV/sec, with peaks scaled between 0 and 20 mV, depending upon the noise level. The alternative test is the absolute value of the signal exceeding a preset threshold, which is useful only where the baseline is extremely constant. In some commercial integrators a combination of the two criteria is used.

When a peak has been detected, integration begins at a point slightly before the peak detection point, as shown in Fig. 8. This ensures that the whole peak is integrated without any small initial loss. Integration is carried out in most cases by a trapezoidal method which is satisfactory if at least 20 points span the peak, but more elaborate integration methods can be used for very slightly improved precision if required and if justified by the precision of the other factors in the analysis. A number of different criteria can be used for detecting the end of a chromatographic peak, either a signal level threshold, or a slope criterion, or again a combination of both. The author has found

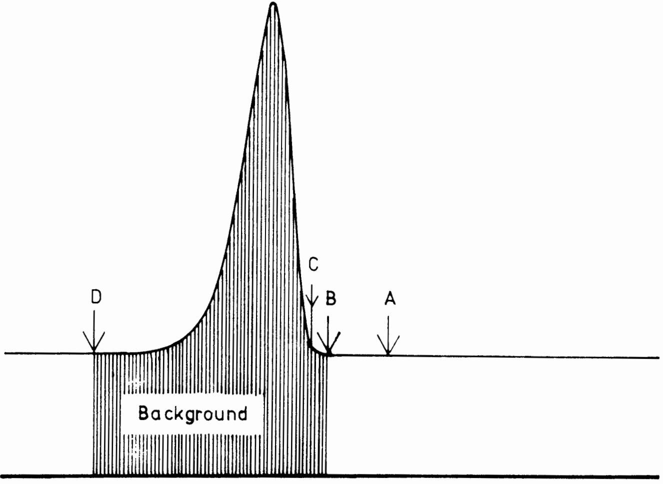

Figure 8 The events occurring during the integration of a single, fully resolved peak. (A) Peak sensing start. (B) Integration start. (C) Peak detected. (D) End of peak detected.

that the slope of the signal falling to a value less than 1.01 × the current baseline slope is a useful end-of-peak detection criterion, provided, of course, that end-of-peak detection can occur only after the peak maximum has been detected.

Finally, it remains necessary to subtract the background signal from beneath the peak, and the simplest way of doing this is to join the point at which a peak was detected and the point at which integration was terminated with a straight line and to evaluate the background as the resultant trapezium. If the baseline drift is only slight, as it should be in quantitative analytical chromatography, this approximation is entirely adequate for the determination of precise peak areas. Even where the baseline is falling rapidly, it is often the best possible approximation.

The above description applied, of course, to the integration of separate, perfectly resolved peaks, and was subjected to a rigorous test by the author [2]. Commercial integrators are much more elaborate in their software, to take account of any combination of partially resolved peaks. The programming effort required to enable an integrator to produce a result with any conceivable shape of chromatogram is hardly justified in a laboratory in which the chromatograph can be adjusted to give separations worthy of precise quantitation. Simply because an advanced program can produce a result whatever the chromatographic separation, the precision and ultimate meaning of that result is severely reduced unless good separation is achieved.

The manner in which the instrument presents the results has a bearing on this: if the instrument simply presents a table of numbers, the chromatographer is liable to accept these without proper inquiry into the precision attached to them. Computer-controlled chromatographs can be programmed to assess the precision of their measurements, but this is not usually incorporated in commercial instruments. For example, in the Pye-Unicam Series PU4000 Liquid Chromatographs, results are presented with between 5 and 6 significant figures, implying a precision of 1 part in 10^5. For almost all quantitative chromatography, precision is hardly likely to be better than 1 part in 1000. It is, therefore, important that some visual record of the chromatogram is readily available, either on a recorder, or preferably on the printed output from the computer where it is inseparable from the numerical data. In this way the chromatographer always has in hand an estimate of the quality of the chromatographic separation upon which the calculated analysis depends.

V. THE SELECTION OF AN AUTOMATIC LIQUID CHROMATOGRAPHIC SYSTEM

Having reviewed the anatomy of an automatic liquid chromatographic system, and the manner of operation of its components, it remains to consider the criteria for the choice of such a system. Apart from the wide range of commercially available instruments, (reviewed recently in [1]), the chromatographer has to choose the level of automation required. Table 3 shows liquid chromatographic instrumentation divided into four levels of automation and complexity. The simplest liquid chromatograph consists of a solvent reservoir, pump, column, and detector, and the construction of such a system from purchased components allows a great deal of the analytical work of more elaborate instruments to be carried out with the minimum of expense.

By far the greatest contribution to liquid chromatographs has arisen from the addition of some computing facility to the above simple system. This allows the automatic calculation of retention data and the integration of peak areas; very simply provided integrations are restricted to analyses yielding fully resolved peaks. This first level of automation might be realized using a microcomputer and an A/D convertor as described earlier, as in the second stage of automation, by the use of a general-purpose computing integrator. The main advantage of the former of these is the low cost and complete flexibility for calculation and results, for data storage, and for presentation. The system can be designed and programmed for a particular repetitive analysis, and the results presented in the most convenient manner. The disadvantage is that some programming knowledge is necessary to set up the system, and if the interfaces are constructed in the laboratory, modest electronic workshop facilities need to be available. The

Table 3 Levels of Automation in Liquid Chromatographs

Level of automation	Instrumentation	Applications	Examples
1.	Single solvent, single manually controlled pump, detector, interface, and microcomputer.	Rapid, simple analyses of easily separable mixtures at low cost.	Laboratory-constructed instrumentation as described in text and in Refs. [2,5].
2.	Single or dual solvent manually controlled pump, detector, and computing integrator.	More complex mixtures analyzed with integration of partially resolved peaks.	As above but with computing integrator such as Laboratory Data Control LDC301.
3.	Dual or ternary solvent system with computer-controlled pump, and computer-interfaced detectors.	Analysis of complex mixtures with quantitative precision and separations requiring optimized polarity programs with high reproducibility.	Applied Chromatography Systems Model 300 Hewlett-Packard 1084B Spectra-Physics SP8700.
4.	Dual or ternary solvent system with computer-controlled pump, computer-interfaced detectors, and automatic sampling system.	Fully automatic repetitive analysis of complex mixtures for use where a large number of similar separations must be achieved as rapidly as possible.	Pye Unicam PU4000 series Varian 5000 and Vista series.

advantage of the commercial computing integrator is that the relatively difficult job of programming the integrator to cope with partially resolved peaks has already been carried out and the instrument can be used by an operator without computer skills. But in either case there can be no doubt that if a large number of quantitative analyses are to be carried out, the facility for automatic data gathering, peak integration, and data storage and presentation is essential.

Following the computing integrator closely in priority is the automation of the pump (stage 3 in Table 3). If all the analyses are to be carried out isocratically, then a single, manually controlled pump of any convenient type can be used, but if reproducible polarity programs are to be generated, and the solvent program repeated frequently, the computer-controlled pump becomes essential. The automation of the pump in this way results in highly reproducible polarity programs, the profiles of which are precisely specified. In addition, the automatic restoration of the original mobile-phase composition after the development of the chromatogram assures rapid and convenient operation. In selecting the appropriate solvent delivery system, the user must also consider the solvent systems which will be employed. For most purposes, a dual-pump system with two solvents is used, but instruments are produced which accommodate three or more solvents. A further factor is the corrosive character of the proposed solvent system, as, for example, in some ion-exchange chromatographic separations, solvents of low pH are used, and the pumping system needs to be resistant to them. Automation at stage 3 appears to be the most satisfactory level for research purposes. The requirements are that the operating conditions be finely adjustable and highly repeatable, and that quantitative measurements be attended with a high degree of precision.

If a very large number of samples require analysis of a routine kind, it is clearly beneficial to carry out analysis continually, and in this case an automatic injection system is necessary (see Table 2, stage 4). Most commercially available automatic injectors have the capacity for some 100 samples in a single loading, and clearly this facility is available only on instruments which also have automatic pump control, and data gathering and storage facilities.

Having identified the level of automation required, a number of other criteria will enable the selection of a suitable instrument from the appropriate range. Among these criteria is the availability of a column oven in cases where higher than ambient temperatures are required for a separation. The choice of detector is, of course, a matter of primary importance, and in cases where its parameters are adjustable, it is necessary to decide whether these should be under microprocessor control or not. Clearly, if a number of different analyses are to be carried out automatically, each requiring a different wavelength setting on a UV absorption detector, control of the monochromator from the computer is essential.

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COMBINED LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY OF DRUGS

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I. INTRODUCTION

Mass spectrometry, because of its high sensitivity and specificity, provides a unique method for the characterization and determination of a large number of organic compounds. A mass spectrum provides a fragmentation pattern which is usually characteristic of a particular compound. Mixtures of compounds provide overlapping spectra and require prior separation of individual components before they can be characterized.

Combined gas chromatography-mass spectrometry (GC-MS) is a well-established method for mixture analysis [1]. The gas chromatograph is used to effect prior separation of the components of a mixture before they enter the mass spectrometer. Unfortunately, GC has certain limitations in that it is not well suited to handle compounds that are of low volatility and/or thermally labile. Although in many cases these problems can be overcome by derivatization [2], sometimes it is difficult to find a suitable derivative. Derivatives may be thermally labile, and sometimes the reactions used give unexpected products.

One of the major advantages of high-performance liquid chromatography (HPLC) is that it can be used to study many compounds which are not amenable to GC. This covers a large number of biologically active compounds, such as peptides, antibiotics, drugs, and toxins. Thus HPLC provides an ideal method for their separation from complex mixtures such as biological fluids. The use of a mass spectrometer

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(MS) as a detector for HPLC (LC-MS) provides a very specific method for the characterization of compounds, and also the MS is a more universal HPLC detector than any of the others available.

Many reviews of the LC-MS combination have been written [3-6], and there is a wide area of application including many problems related to the determination of biologically active compounds. Specific areas of interest include bioassays of drugs in plasma, urine, and other biological fluids. In addition, LC-MS is potentially very useful for the identification of drug metabolites and also for screening of biological matrices for unidentified compounds in cases of overdose and poisoning.

Many applications of LC-MS have been published (e.g., [5]). There are many more problems in the field that could be solved by LC-MS if the instrumentation were available in the laboratory. There are a number of reasons why LC-MS is not as routinely available in analytical laboratories as GC-MS. The coupling of an HPLC to an MS is not as straightforward as that of GC-MS, and it is unlikely that LC-MS will ever supplant GC-MS for the analysis of thermally stable compounds of adequate volatility.

There are two major difficulties to be overcome before LC-MS can be accomplished. First, a conventional HPLC system delivering approximately 1 mL min^{-1} of solvent produces 150-200 atm mL min^{-1} of gas volume, whereas a conventional mass spectrometer is only capable of handling up to 20 atm mL min^{-1}. Second, it may be difficult to obtain good mass spectral data on thermally labile and/or involatile compounds. As a result of these difficulties, a number of research groups have implemented off-line methods for LC-MS. However, the advantages of an on-line system are numerous, and a number of LC-MS interfaces have been developed adopting a variety of approaches, and LC-MS interfaces are commercially available. The major recent developments in the fields of off-line and on-line LC-MS interfacing are covered in the following text, together with some applications, expecially where related to biologically active compounds.

II. OFF-LINE LC-MS

Off-line collection of samples from an HPLC system for subsequent mass spectral analysis has certain advantages. There is no restriction on the HPLC solvent system, and it is the only method which can be used for compounds that do not provide useful mass spectral data by either electron impact (EI) or chemical ionization (CI), and have to be examined by other softer methods of ionization such as field desorption (FD-MS) [7] or fast atom bombardment (FAB) [8]. However, some of the more recent developments in on-line LC-MS, which will be considered later, implement alternate ionization modes which might be able to provide data on such compounds. Off-line LC-MS is, however, the

method of choice for many laboratories that do not possess an LC-MS interface, either because they lack the funds to purchase one or remain to be convinced of the viability of the technique from the commercially available interfaces.

The methodology for off-line LC-MS has been reviewed [9] and semiautomated systems developed [10]. One system involving an automated probe has been developed [11], and also an approach using a rotating disc interfaced to a time-of-flight mass spectrometer [12-14]. The latter approach is covered in greater detail in Sec. III.B.3. Recently a semiautomated system for the combination of a microbore HPLC system (micro LC) to a mass spectrometer was reported [15].

The off-line combination of HPLC and FD-MS has been used in the determination of drugs and other compounds of pharmaceutical interest in biological fluids and natural products [16]. Purification is accomplished by HPLC, and identification and quantification by FD-MS. In general, stable isotope-labeled analogues are used as internal standards for quantification. Their identical chemical behavior means that they will co-chromatograph with the sample and can be sampled in the same fraction of eluent. This method has been successfully used for the identification and quantification of the anticancer drug cyclophosphamide and its metabolites in body fluids of patients with multiple sclerosis, and also for the identification of barbituates in body fluids and tissues [16]. These examples illustrate that off-line LC-MS, particularly when using FD-MS, can produce very successful results. However, it seems likely that similar results could be achieved in the future using on-line LC-MS.

III. ON-LINE LC-MS

The advantages of the direct coupling of an HPLC to a mass spectrometer for the determination of drugs in biological fluids would appear obvious. Purification of samples is accomplished on-line, and when one is attempting to identify unknown compounds such as drug metabolites, mass spectral data allowing at least partial characterization are readily available. However, as was previously stated, certain difficulties have to be overcome before successful on-line LC-MS can be accomplished.

A. The LC-MS Interface—Introduction

The major incompatibility of the LC-MS combination is the difference in the amount of gas pressure produced by the HPLC effluent as compared to that which the mass spectrometer can handle. Another major consideration is that there must be little or no loss of chromatographic integrity in the interface, resulting in loss of resolution in the HPLC separation, and this is especially important when micro LC is being

used. To overcome this, an interface must have little or no dead volume.

Ideally, an LC-MS interface should not restrict the operation of either the mass spectrometer or the HPLC system. However, each of the interfaces so far developed restricts one or both to some extent. The methods which have been used to accomplish LC-MS can be divided into three major types of interfaces, and a review of each of these approaches including relevant applications is given in the following sections.

B. Transport LC-MS Interfaces

In this approach, the HPLC effluent is mechanically transported into the ion source of the mass spectrometer. The majority of the HPLC solvent is removed prior to entering the mass spectrometer, thus allowing either EI or CI mass spectra to be obtained. Most of the work in this area has been carried out using the moving-belt LC-MS interface (*1*), and commercially available interfaces have been developed. Another interface involving continuous preconcentration of the HPLC effluent has been developed (*2*), and preliminary results have been reported for a new approach involving an interface for combined liquid chromatography/time-of-flight spectrometry (*3*).

1. Moving-Belt LC-MS Interfaces

This type of LC-MS interface and its applications have been reviewed recently [17]. The initial studies were carried out using a modified Pye Unicam moving wire HPLC detector [18] in which the transport wire passed through differentially pumped vacuum locks into the ion source of a quadrupole mass spectrometer. Since the solvent was removed in the vacuum locks, both EI and CI mass spectra could be recorded. A major limitation was that less than 1% of the eluent could be transported into the mass spectrometer. However, this situation has been improved by the development of a moving-belt interface [19]. Initially stainless-steel belts were used [19], but these have generally been replaced by polyimide Kapton belts [20] which provide a more inert surface.

Figure 1 shows the basic design of the first commercially available moving-belt interface (Finnigan-MAT). HPLC eluent passes through a low dead-volume splitter and onto the belt. It flows under an infrared heater which vaporizes most of the solvent, and then through two vacuum locks to remove the remainder of the solvent. The belt passes into the ion-source region where the sample is flash-vaporized off the belt and into the ion-source chamber. The belt then passes back via a cleanup heater, which removes any residual sample, and the scrubber, which can be used to remove any involatile material.

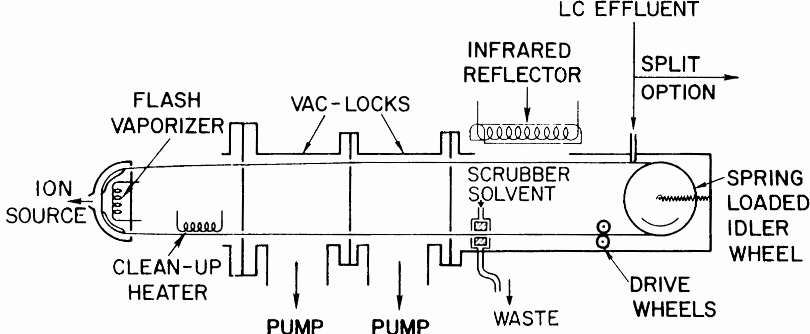


Figure 1 Longitudinal section through early Finnigan-MAT LC-MS interface.

There are two more recently developed commercial systems (VG Analytical [21] and Finnigan-MAT [22]), which work on the same principle. The major differences from the earlier system are that in both cases the belt actually passes into the ion source of the mass spectrometer, and also the interfaces are available on both quadrupole and magnetic instruments. The positioning of the interface inside the ion source should result in enhanced sensitivity and might help obtain mass spectra from more difficult compounds. Few results have been published using either system, making it difficult to evaluate them, other than in principle. One group has used both the VG LC-MS interface and the earlier Finnigan-MAT interface [23,24] and recently reported that although the VG interface appeared better for CI, the Finnigan-MAT interface gave better results for EI [24].

A number of difficulties have arisen using the moving-belt interface design. The major problem has been handling HPLC solvent systems with a high percentage of water (50%). Small droplets form on the belt instead of the normal continuous film, which leads to serve fluctuations of the vacuum in the ion source. A number of attempts have been made to improve this situation: placement of heaters in the vacuum chambers [25], addition of a second water-miscible solvent, e.g., 2-propanol, onto the belt prior to the HPLC eluent [26], or use of a wetting agent [27] have all contributed to improve performance. A system using a preheated argon stream to produce an aerosol deposition of eluent on the belt has been employed [28], and also a modified segmented flow extractor [29,30]. In the latter case, the solute is extracted from the aqueous system into a suitable organic solvent, which is then delivered onto the belt. This approach has the advantage that solvent systems using ion-pairing agents may be used, but loss of sensitivity and chromatographic integrity may result. The use of micro LC-MS also allows handling of high-percentage aqueous

solvent systems due to the reduced eluent flow-rate [31-33]. Background interference from residual solvent ions may cause some difficulties and restrict the available LC-MS mass range to greater than *m/z* 110. However, micro LC-MS should help overcome this since less solvent is being introduced.

The major advantage of the moving-belt LC-MS interface is that both EI and CI data are available. Differences have been reported between the spectra of sugars obtained from on-line LC-MS and those obtained by spotting solutions of the same samples onto the moving belt. The latter appears to give more abundant ions in the molecular weight region [17,34]. Also, EI LC-MS spectra obtained from ergot alkaloid samples at low concentrations appeared to suffer thermal degradation, which was not seen at higher concentrations, under CI conditions or when spectra are obtained from the direct insertion probe [35].

Moving-belt interfaces have been reported for use with a plasma chromatograph [36], secondary ion mass spectrometry and laser desorption [37-39], and, more recently, fast atom bombardment [40]. One report used the thermospray technique (Sec. III.D.*2*) to deposit nonvolatile samples onto the belt [41]. The solvent was vaporised, and samples were transported into the ion source of a laser desorption quadrupole mass spectrometer. In another report, a continuous stainless-steel belt perforated with small holes was used [42]. The solvent was removed in an evaporator and the solute converted to hydrocarbons by catalytic reduction. These hydrocarbons produce spectra characteristic of the solutes, and impressive analyses of complex lipid mixtures were reported. The major disadvantage of this system is that conventional EI and CI data are not obtained.

Maintenance of chromatographic integrity is very important in any HPLC system, and it was recently well documented that there is little loss of resolution using the belt interface [43]. The best results were obtained using a concurrent preheated nitrogen stream to direct flow onto the belt.

Applications: There have been many reports published using moving-belt LC-MS interfaces for the analysis of biologically active compounds: [5,17,24,26,27,29-33,41,42,44-55]. For example, the metabolism of the herbicide chloropropham (CIPC) in rats and paddy plants has been studied by LC-MS [5,33], and quantification of the antiarrythmic agent disopyramide [1] has been attempted [50]. Low nanogram detection

$CONH_2$ N $CH(CH_3)_2$ $CH(CH_3)_2$ N

1

limits for disopyramide were obtained by selected ion monitoring (SIM) in urine and plasma. Figure 2 shows an LC-MS CI SIM trace of an extract of urine from a monkey fed the drug and stable isotope analogues.

Identification and quantification of the antiulcer agent ranitidine [2] and three metabolites has been accomplished by LC-MS [55a].

$$(CH_3)_2NCH_2\text{-[furan]-}CH_2SCH_2NHC(=CHNO_2)NHCH_3$$

2

Figure 3 shows the reconstructed total-ion-current (TIC) trace and positive CI (PCI) mass spectra obtained from an extract of human urine collected after administration of ranitidine. Quantification was carried out by SIM using [$^2H_3$]ranitidine as an internal standard, and detection limits of 10 ng or 1 μg/ml of urine were obtained. Improved LC-MS results for ranitidine have been recently reported [55b].

The sensitivities obtained do not appear as good as GC-MS. However, insufficient work has been reported in this area to form any conclusions, and the improved construction of the new Finnigan-MAT interface may afford increased sensitivity. Enhanced sensitivity has also been reported using micro LC-MS [32,33].

2. *Continuous Sample Preconcentration LC-MS Interface [56-59]*

This interface was first reported in 1979 [56] and is now commercially available (Extranuclear Laboratories Inc., Pittsburgh, Pa.). It adopts a different approach to the moving-belt system, and Fig. 4 shows the basic construction.

The HPLC effluent is concentrated by evaporation as it flows down an electrically heated wire in a stream of warm nitrogen. The concentrated effluent flows through a capillary tube to a very small needle valve, which regulates the flow into the mass spectrometer. Since most of the HPLC effluent is removed before the sample enters the ion source, EI and CI mass spectra may be obtained.

Limited success has been reported with this interface, although the determination of the anticonvulsant valproic acid in human serum has been reported [58]. These are only preliminary results, and further work would have to be done before the potential of this interface can be properly assessed.

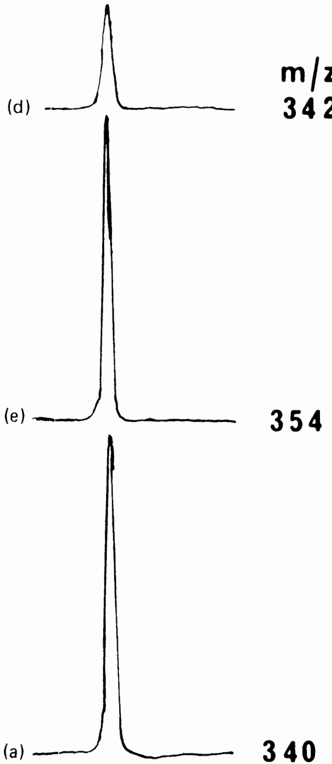


Figure 2 LC-MS PCI (positive CI) SIM of an extract of urine from a monkey fed with disopyramide (a) and [^{13}C, ^{15}N]disopyramide (d), and drug (a) with [$^{2}H_{14}$]disopyramide (e) as internal standard. LC conditions: column, 20 cm × 4.5 mm 5 μm Partisil; 1 mL min^{-1} methanol: *t*-butylamine:acetic acid (98:1:1); 30% split to LC-MS interface. (Reprinted with permission, from Ref. 50.)

3. Combined Liquid Chromatography/Time-of-Flight Mass Spectrometry [12-14]

This LC-MS method was developed to utilize ^{252}Cf plasma desorption mass spectrometry (PDMS) as a detector for HPLC. This mode of ionization has the advantage that it can be used to produce mass spectra from thermally unstable compounds where direct heat exposure should be avoided.

The PDMS interface can be used for both on-line and off-line LC-MS [13]. HPLC effluent is introduced through a capillary tube into a vacuum chamber. The tip of the capillary may be gently heated to

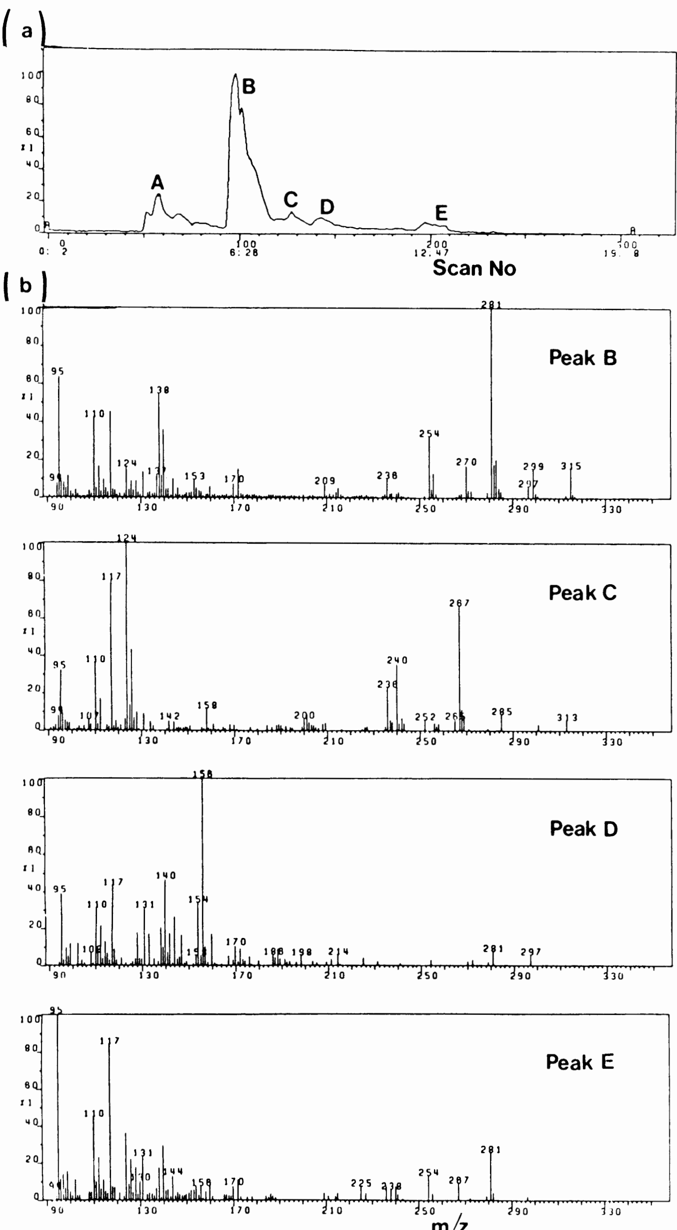


Figure 3 (a) TIC-obtained ammonia PCI LC-MS of a human urine extract, collected 0-2 hr after oral administration of ranitidine. LC conditions: column 10 × 5 mm S5N CN; 0.5 mL min^{-1} methanol:propan-02-01:5M ammonium acetrat (50:50:1). (b) Ammonia CI mass spectra from TIC peaks: B = ranitidine; C = desmethylranitidine; D = ranitidine-*S*-oxide; E = ranitidine-*N*-oxide. (Reprinted with permission from Ref. 55.)

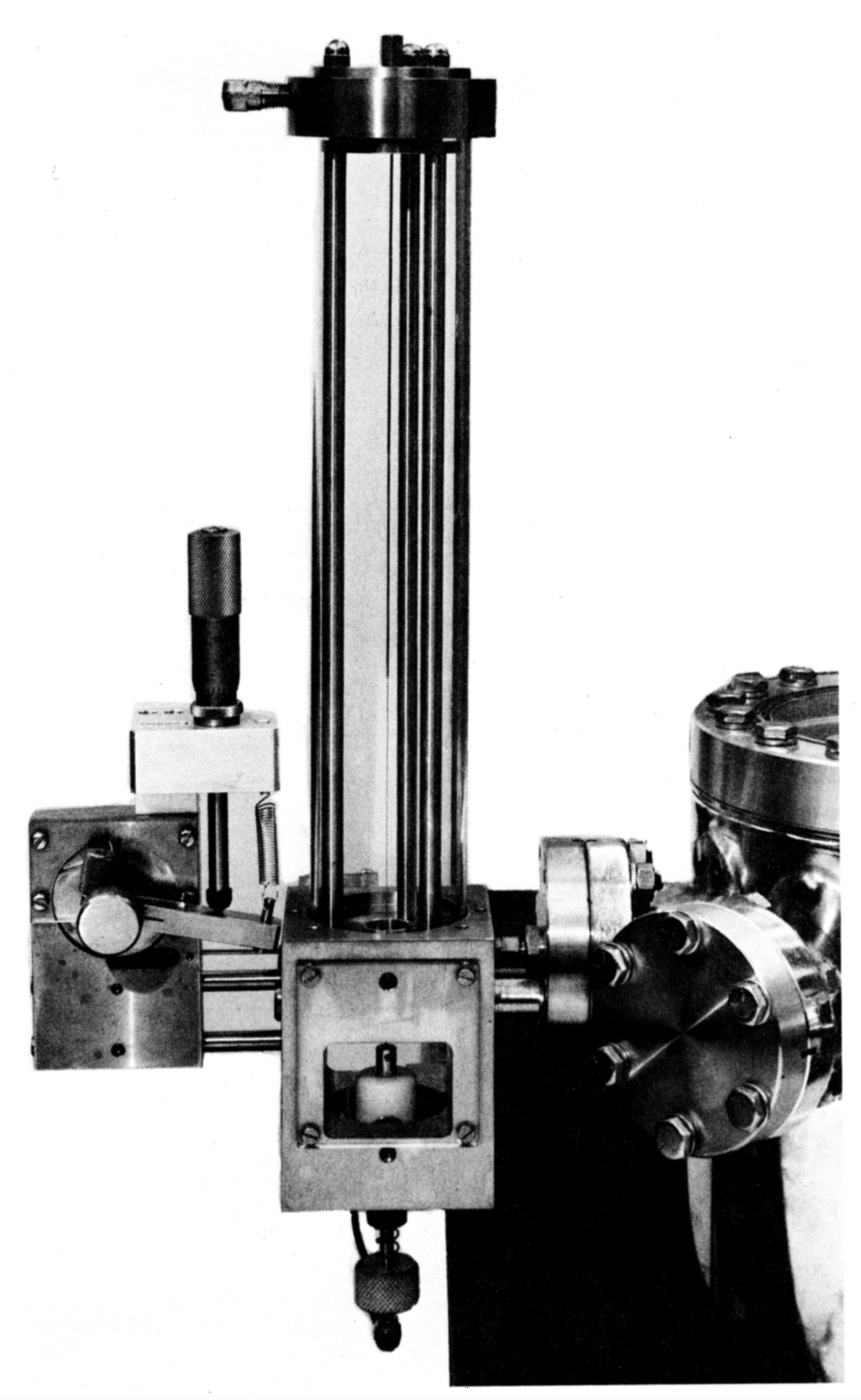


Figure 4 Extranuclear continuous sample preconcentration LC-MS interface.

avoid plugging due to frozen solvent. The effluent expands and is collected as a thin layer on a sample foil where it freezes instantly. As the eluent vaporizes, all nonvolatile compounds are collected in a vacuum drying process with little heat irradiation involved. Twelve sample foils are mounted on a disc which is rotated in steps into the mass spectrometer, allowing twelve fractions of effluent to be consecutively collected and analyzed at approximately one sample per minute.

Determination of the antiarrhythmic drugs quinidine and verapamil, the antineoplastic agents Etoposide, Teriposide, and three *Vinca rosea* alkaloids has been reported [13,14]. This work was carried out with standard compounds spiked into serum for the highly toxic antineoplastic agents, and verapamil was quantified after intravenous injection into a human volunteer.

Although these preliminary results appear promising, further development of the interface is required to fully assess its merits. Distribution of effluent into twelve fractions was sufficient in the cases examined, but further applications would require and increased number of fractions, and preferably a continuous sampling system would be desirable. An interesting possibility is the potential for combining this LC-MS system with laser-induced mass spectrometry (LIMS) and secondary ion mass spectrometry (SIMS), with the possibility for combining all three methods in the same time-of-flight mass spectrometer.

C. Direct Liquid Introduction (DLI) Without Sample Enrichment

In this type of LC-MS interface, the HPLC effluent is introduced directly into the ion source of the mass spectrometer. This can be accomplished in three ways. In the first approach the mass spectrometer is altered so that it can tolerate large gas volumes, and this is accomplished using atmospheric pressure ionization (*1*). In the second approach, only a portion of the HPLC effluent is introduced into the mass spectrometer (*2*). This percentage is dictated by the amount of vapor pressure the mass spectrometer can handle. In the third approach the HPLC system is modified utilizing reduced flow-rates, that is, micro LC (*3*). When the flow-rate is reduced to 10-50 μL min^{-1}, the pumping system of a CI mass spectrometer can handle all the HPLC effluent.

1. Atmospheric Pressure Ionization (API)

The API mass spectrometer operates at atmospheric pressure and thus is able to tolerate the large gas volumes generated by vaporization of the HPLC effluent. A number of LC-MS systems have been designed using this approach. The solvent vapor is ionized by a ^{63}Ni or corona

discharge [60-62], and in one report using a plasma chromatograph [36]. The ions produced then react with sample molecules, and the resulting ions sampled through a pinhole aperture into the mass spectrometer analyzer.

In initial studies, mixtures of steroids and drugs were examined. However, use of this system was restricted because of a number of problems associated with API. Samples were vaporized at atmospheric pressure, and this resulted in difficulties analyzing compounds of low volatility. Thus some method of sample vaporization prior to introduction into the mass spectrometer is necessary. Another problem encountered was that usually only $[M + 1]^+$ or $[M - 1]^-$ formation occurred, severely limiting the amount of structural information available. This could be overcome by introducing a collision gas into the system and inducing fragmentation by collision-induced dissociation (CID) [63]. Other problems found in initial work were the formation of polar solvent cluster ions which made it difficult to characterize compounds of low molecular weight, and also the system tended to be insensitive to compounds which did not have a high electron affinity.

Recently work has been carried out using a different API mass spectrometer utilizing various methods of sample introduction and ionization [64,65]. A technique known as "ion evaporation" is used in which extraction of ions from solution occurs; this method is discussed in Sec. III.D.*2*. A DLI LC-MS interface (see below, Sec. *b*) has been interfaced to a similar API mass spectrometer with a triple quadrupole analyzer [66]. This allows both CID and MS-MS data to be obtained, and some practical LC-MS and LC-MS-MS results have been obtained on the determination of sulfa drugs in biological fluids (Sec. IV).

2. Direct Introduction of a Portion of the HPLC Effluent

This is the simplest approach to LC-MS. The HPLC effluent is split, and only that amount of solvent which can be tolerated by the mass spectrometer is fed into the ion source. The first studies using this approach were carried out under EI conditions [67]; however, less than 0.1% of the sample was utilized, and this appeared impractical. If the mass spectrometer is operated under CI conditions, higher source pressures can be used, and approximately 10-60 $\mu L\ min^{-1}$ of liquid can be introduced [68-69]. The eluent is used as a CI reagent gas; it is ionized in the ion source and generates ions from the solute by ion-molecule reactions.

The simplest design is a drawn-out glass capillary tube passing directly into the ion source [23,68-73], and a large number of drugs have been studied using this system [70]. Difficulties are experienced with this type of interface because low-volatility solutes may be deposited in the capillary tube due to prior evaporation of the solvent, causing blockage. In early designs, removal of this blockage involved

reforming the capillary tip orifice. However, in a more recent design, capillaries were made in a batch system and could be replaced in 10-15 min [73].

These difficulties may be avoided by replacing the capillary tube with a diaphragm pinhole orifice placed directly adjacent to the ion source. The total LC effluent flows past this orifice while a small portion sprays into the ion source. Two commercially available LC-MS interfaces have been produced based upon this approach (Hewlett-Packard, Palo Alto, Calif. [74,75] and Nermag, France [76]). In both cases the pinhole orifice consists of a centered small hole approximately 2-5 μm in diameter in a replaceable metal diaphragm. Cooling water is supplied to the probe tip to prevent vaporization of the solvent before it enters the ion source. The basic construction of the Hewlett-Packard DLI LC-MS system is given in Fig. 5. Liquid-nitrogen cryogenic pumping is supplied with the Hewlett-Packard interface to supplement normal CI pumping.

Much work has been carried out in order to ascertain optimal instrument parameters for operation of these types of LC-MS interfaces [76-81]. Due to the nature of the interface, plugging of the diaphragm may occur if solvents or samples contain particulate matter, or if a

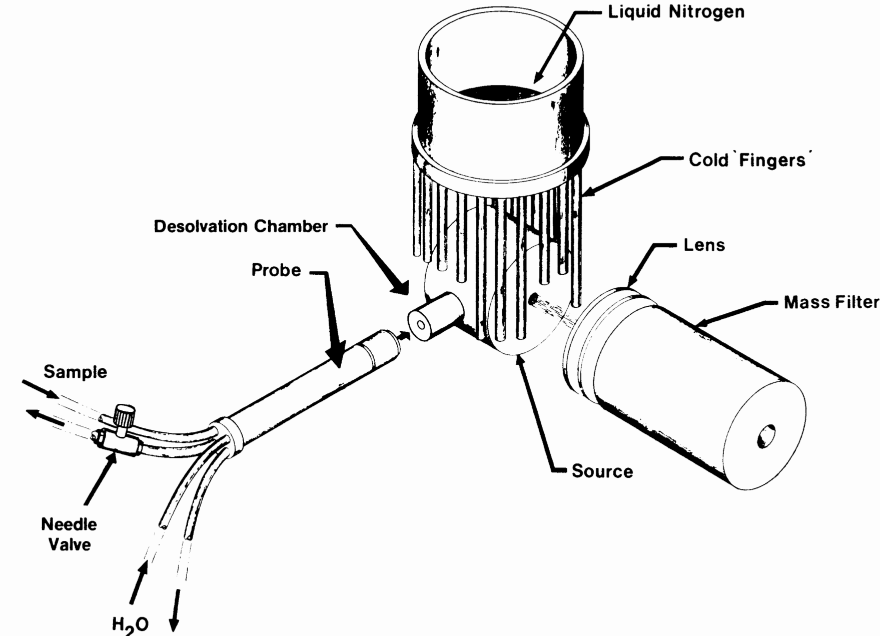


Figure 5 Schematic of direct liquid introduction LC-MS interface (Hewlett-Packard).

filter is not included in the system after the HPLC column to trap particles that may evolve from the column.

The overall sensitivity of this type of interface is not high since only approximately 1% of the total HPLC effluent is being analyzed. This may be overcome by interfacing to a micro LC system, allowing all the effluent to be analyzed; this is described in Sec. III.C.*3*. Another disadvantage associated with this approach is that one is limited to solvent-mediated CI spectra. This restricts the choice of solvent since not all are equally efficient CI reagent gases. Also, one is generally limited to compounds of molecular weight greater than 150 due to interference from solvent cluster ions. However, recent modifications of the interface have been reported which have the facility of breaking these cluster ions and thus offer the possibility of starting an aquisition at 60 amu [81].

Applications: DLI LC-MS interfaces have been used in the determination of a wide number of biologically active compounds [55,70-72,80a, 82-97]. The Nermag LC-MS interface has been used in the determination of marine sterols [91], cannabis leaf extracts [5], and recently an NCI LC-MS spectrum of vitamin B_{12} (molecular weight, 1354) was reported [80a]. The vitamin B_{12} data are impressive since an abundant ion at *m/z* 1353 corresponding to $[M-H]^-$ was observed.

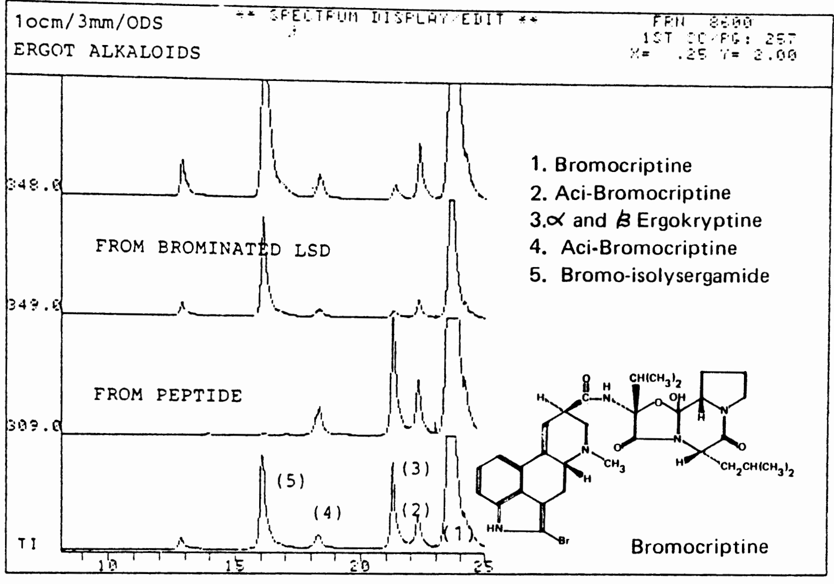
The Hewlett-Packard LC-MS interface has been used in the determination of triazine herbicides [96] and the identification of strychnine in the stomach contents of a poisoned dog [76]. Ergot alkaloids, such as lysergic acid diethylamide, in urine have been examined by LC-MS using this interface [5,86], and also the more complex ergot peptide alkaloid ergokryptine and the semisynthetic prolactin inhibitor bromocriptine in pharmaceutical preparations [86-88].

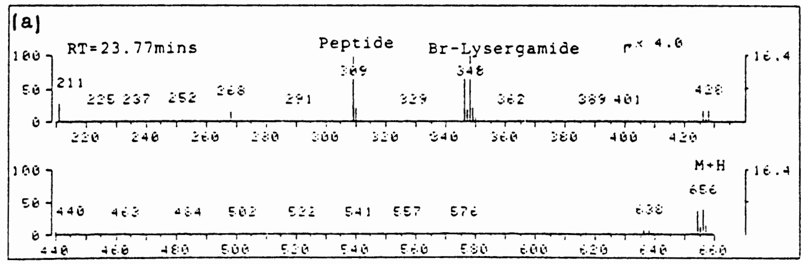
Figure 6 shows LC-MS analysis of an ergot alkaloid mixture and positive CI LC-MS spectra of bromocriptine and ergokryptine. These alkaloids cannot be analyzed by GC-MS due to their low volatility and thermal instability. Thus, LC-MS provides an ideal method for their determination.

3. Direct Liquid Introduction Micro LC-MS

The use of micro LC presents a natural progression from conventional HPLC for LC-MS interfacing. Decreasing the flow-rate decreases the amount of gas volume being produced, one of the major problems of LC-MS interfacing. For DLI LC-MS, reducing the flow-rate allows all or most of the HPLC effluent to be introduced into the mass spectrometer and should markedly increase the overall LC-MS sensitivity.

Packed [98] and open-tubular [98,99] capillary columns, and microbore columns [100,101] have been used for micro LC. Performance and instrumentation for micro LC have been covered in many reports and reviews (e.g., [102]). Micro LC-MS has been carried out using





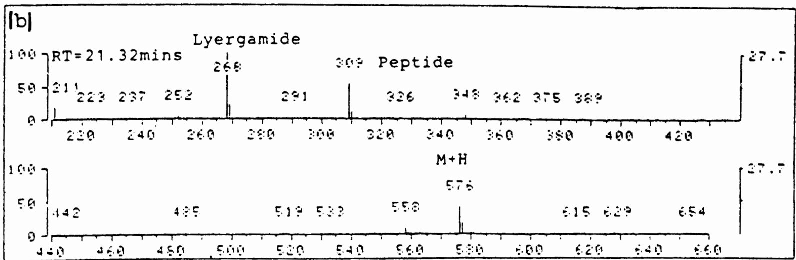


Figure 6 PCI LC-MS, total-ion-current trace, and mass chromatograms of an ergot alkaloid mixture. LC conditions: column, 10 cm × 3 mm ODS; 0.5mL min^{-1}, acetonitrile:water:triethylamine. (a) PCI LC-MS spectrum of bromocriptine. (b) PCI LC-MS spectrum of ergokryptine. (Reprinted with permission from Ref. 84.)

packed microbore columns [23,33,82,85,86,89,92-97,103-106]. These columns are commercially available [107], and much of this work has been carried out using the Jasco micro liquid chromatographic system [108].

DLI LC-MS interfaces have been developed especially for micro LC-MS [85,89,96,103-106] since the dead volumes in the commercially available DLI LC-MS interfaces (Sec. III.C.*2*) are too large to be compatible with micro LC. Considerable success has been obtained using a design based on the original Hewlett-Packard LC-MS interface. The basic design is given Fig. 7. It consists of a narrow-bore (approximately 0.004 i.d.) tube leading from the column exit to a diaphragm with a centered pinhole orifice through which the total micro HPLC effluent is sprayed into the ion source of the CI mass spectrometer.

This interface has been used in the determination of the diuretic trichlormethiazide in equine urine extracts [94,95]. Full-scan mass spectra of 1.25 ng of trichlormethazide were obtained. Standard antibiotic mixtures have also been examined and identified in a crude antibiotic fermentation broth [92,97]. Figure 8 shows the UV chromatogram and NCI ion current profiles from the micro LC and micro LC-MS analysis of an extract of equine urine after administration of the steroid betamethasone [97]. Betamethasone [3] and its major metabolites were identified in the extract.

3

Another DLI Micro LC-MS interface has been designed with the exit of the column directly abutting the diaphragm to further minimize deal volume [89]. However, this was further modified to incorporate a splitting system so that higher flow-rates could be used. In another system, the end of the microbore column was introduced directly into the ion-source block [102].

The use of micro LC appears to overcome many of the problems associated with DLI LC-MS, and impressive results have been obtained with "homemade" interfaces. Micro LC also overcomes some of the problems associated with the moving-belt LC-MS interface (Sec. III.B.*1*) and appears to be the method of HPLC best suited for interfacing to mass spectrometry at the present time.

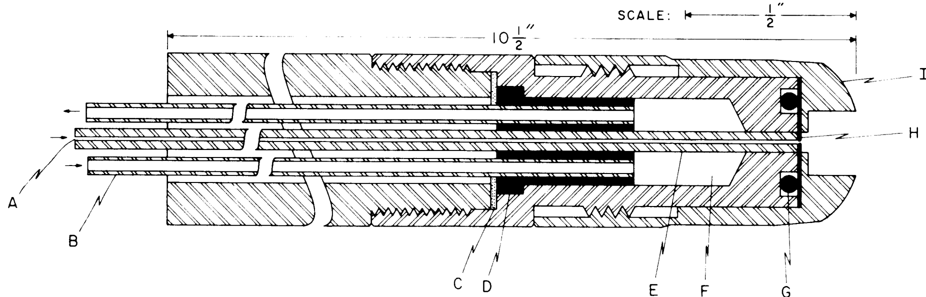


Figure 7 Longitudinal section through DLI micro LC-MS probe, (a) micro LC effluent inlet line, (b) water cooling inlet tube, (c) Teflon washer for maintaining vacuum seal between probe tip/cooling chamber and probe shaft, (d) throughput tube collet, (e) 0.004 i.d. × 0.0625 o.d. stainless-steel throughput tube. (f) water cooling chamber, (g) Kalrez O-ring, (h) diaphragm containing 5-m pinhole, (i) removable end cap. (Reprinted with permission from Ref. 95.)

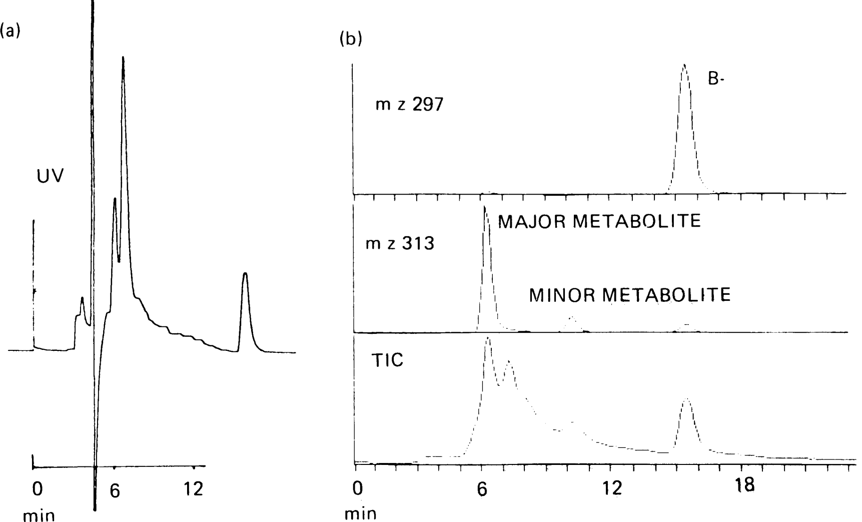


Figure 8 Micro-LC-MS analysis of an equine urine extract, collected 6 hr after administration of betamethasone. LC conditions: column 25 cm × 1 mm ODS-3; 40 μL min^{-1} methanol:water (65:35). (a) UV chromatogram (254 nm). (b) NCI LC-MS TIC plus mass chromatograms. (Reprinted with permission, from Ref. 97.)

D. Direct Liquid Introduction with Enrichment

In this approach, enrichment of solute relative to solvent is accomplished prior to introduction into the ion source of the mass spectrometer. Ideally, enough solvent should be removed to enable EI spectra to be obtained. Initial studies utilize a semipermeable silicone membrane allowing selective diffusion of nonpolar solutes from a polar solvent [109]. Since this is the reverse of normal HPLC requirements and since the interface could be used only with compounds that are volatile up to 250°C, it did not enjoy widespread use.

LC-MS interfaces have been designed based on the jet separator used for GC-MS (*1*), and more recently, impressive results have been obtained using a technique known as thermospray (*2*).

1. Modified Jet Separators

Initial studies were carried out using a modified jet separator [110]. The performance was improved by utilizing a vacuum nebulizing interface. This approach accommodates higher flow-rates and compounds of low volatility, although only solvent-mediated CI spectra are obtianed [111,112]. Many modifications of the nebulizing interface have been reported in an effort to improve performance [113-115]. Generally a jet stream of preheated helium is introduced with the HPLC effluent to produce nebulization and also a system using ultrasonics has been patented [116].

The use of these systems has generally been limited either to micro LC or to use of a splitting system and thus appears to have little advantage over DLI LC-MS interfaces which do not incorporate any method for enrichment (Secs. III.C.*2*,*3*). In one report, two simple interfaces were designed and constructed, one for DLI LC-MS and the other, a nebulizing interface [73]. Performance of both interfaces was found to be basically similar. The DLI interface appeared to suffer from less loss of chromatographic resolution than the nebulizing interface, although the latter should be better for compounds of low volatility.

One of the limiting factors of the DLI with enrichment LC-MS interface is sensitivity, and any method which increases the amount of sample entering the mass spectrometer increases sensitivity. Also, adjusting the nature of nebulizing gas, which can also be used as CI reagent gas, should increase the efficiency of sample ionization and thus sensitivity. Some LC-MS applications have been published using this type of interface. However, its capacity to solve problems which cannot be better dealt with using other methods has not been adequately demonstrated.

2. Thermospray LC-MS Interfaces

In this approach the HPLC effluent is vaporized by heat and vacuum (thermospray) directly into the mass spectrometer. Vaporization is

accomplished by an electrically heated thermospray vaporizer [117-119]. In initial studies, lasers [120-122], sonic radiation [123], and oxyhydrogen torches [124-126] were used to produce vaporization. The latter simpler system was found to be preferable. Stable vaporization and ionization at flow-rates up to 2 mL min^{-1} of aqueous mobile phase have been achieved. The jet of vapor undergoes adiabatic expansion, and a portion passes through a skimmer into the ion source. The excess vapor is pumped away by an auxiliary mechanical vacuum pump.

Conventional EI and CI mass spectral data can be obtained using this system. However, if a mobile phase with a significant concentration of ions in solution is used, no external ionization source is required [125,127-129]. Sensitivities comparable to those afforded by chemical ionization have been reported using this method of thermospray ionization [130]. Reported applications include determination of nucleosides [127,130], amino acids, and peptides [119]. Figure 9 shows the UV chromatogram, TIC (total-ion-current trace), and mass

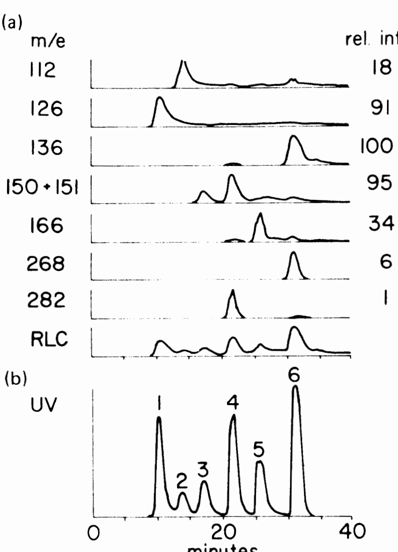


Figure 9 LC-MS analysis of a mixture of nucleosides and bases: 1 = 5-methylcytosine, 2 = cytidine, 3 = 7-methylinosine, 4 = 1-methyladenosine, 5 = 7-methylguanosine, 6 = adenosine. LC conditions: column 25 cm × 4.6 mm Partisil 10 ODS-2; 0.5 mL min^{-1}, ammonium formate (0.02 M, pH 5) for 12 min, then a linear gradient of 100% methanol at 32 min. (a) TIC and mass chromatograms; (b) UV trace (254 nm). (Reprinted with permission from Ref. 124. Copyright 1982, American Chemical Society.)

chromatograms obtained for the LC-MS analysis of a mixture of nucleosides and bases [124]. This experiment was actually carried out using the earlier interface design which incorporated oxyhydrogen torches; however, recent work showed similar results using the thermospray vaporizer [130]. An LC-MS interface based on this principle has recently become commercially available (Kratos Analytical Instruments) [131], and its performance remains to be demonstrated.

The thermospray approach to LC-MS may have definite advantages for the determination of compounds of low volatility. A mechanism for thermospray ionization has been proposed [128,129]. It is believed to be similar to that occurring with other soft ionization techniques such as FAB and field desorption. Thus, perhaps this method could be used for the on-line LC-MS of compounds too unstable to be determined by other LC-MS methods. With more stable compounds it is likely that fragmentation could be induced by interfacing to a mass spectrometer with the capability for CID [63].

A similar ionization process, known as ion evaporation, has also been used to produce an LC-MS interface [64,65], and preliminary results reported include the analysis of amino acids and alkaloids [65]. A similar electrospray technique for mass spectrometry has also been patented [132].

IV. LC-MS, MS-MS, AND LC-MS-MS

In the previous section, the interfacing of an HPLC to a mass spectrometer with MS-MS capability (a tandem mass spectrometer) has been mentioned. MS-MS can be accomplished by either monitoring the decomposition of metastable ions or utilizing CID. In CID, collisions between ions and small gas particles (usually helium) induce characteristic fragmentations. CID has been shown to produce EI-type mass spectra, and it is also possible to produce CID spectra from fragment ions and $[M + 1]^+$ or $[M - 1]^-$ ions produced by soft ionization techniques.

Tandem mass spectrometry can be accomplished by a number of methods including MIKES (mass-analyzed ion kinetic energy spectrometry) [133], linked scanning [134], and tandem quadrupole mass spectrometry [135]. MS-MS has been used to identify unknown compounds in complex matrices without sample cleanup [133]. However, introduction of very crude samples into a mass spectrometer ion source severely decreases source lifetime, and some sample cleanup is advisable. Also, it may be difficult to distinguish compounds of the same molecular weight, such as isomers.

A comparative study of a crude extract of ergot by LC-MS and MS-MS (using B/E linked scanning) has been reported [35]. The MS-MS technique provided a rapid, facile screening technique. Unfortunately, many of the alkaloids were isomeric and could not be

separated, although B/E linked scan spectra of the standard alkaloids provided a method of distinguishing between isomeric pairs. By contrast, LC-MS allowed separation of the isomers, which unfortunately could not be distinguished on the basis of their EI or CI mass spectra alone. These problems would be overcome by an LC-MS-MS combination, whereby both separation and identification could be accomplished. On-line LC-MS-MS would also allow separation of the compound of interest from endogenous material in the sample, which could contaminate the ion source and might produce interfering ions.

Some LC-MS methods, such as DLI LC-MS and thermospray ionization, often produce only [M + 1] or $[M - 1]^-$ ions of compounds. If these techniques were combined with a tandem mass spectrometer, CID could be used to produce a fragmentation spectrum. MS-MS could also be used to separate coeluting HPLC components.

Recently preliminary LC-MS-MS results have been reported using a tandem quadrupole mass spectrometer [66,136]. Sulfa drugs in extracts of equine urine and plasma have been determined using a DLI LC-MS interface on an API triple quadrupole mass spectrometer. Figure 10 shows the results obtained from a equine urine extract. The upper trace is the UV chromatogram, and the lower traces are selected ion profiles resulting from CID of the *m/z* 311 $[M + 1]^+$ ion of sulfadimethoxine [4].

NH_2—C_6H_4—SO_2NH—(pyrimidine with two OCH_3)

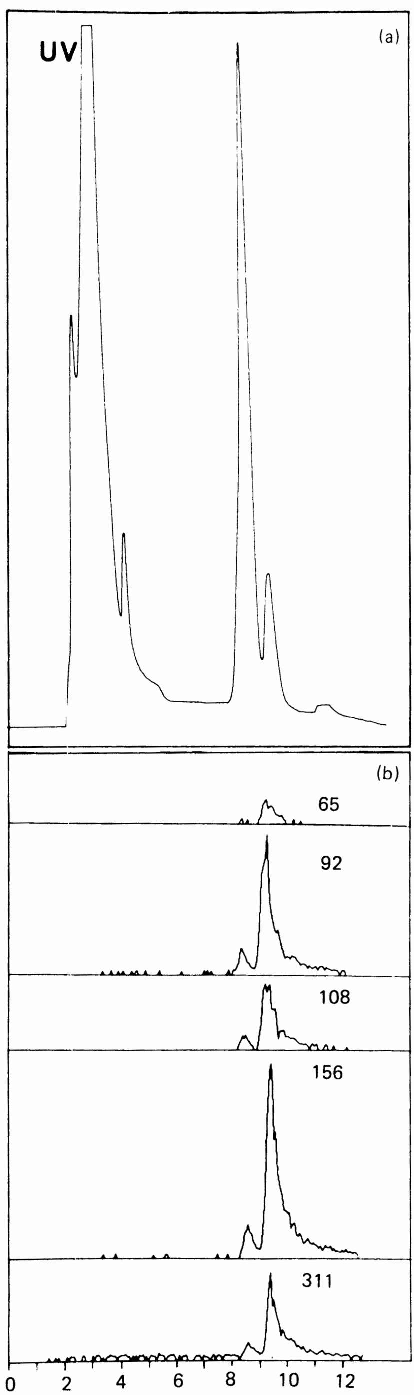
4

These results demonstrate the unique potential of LC-MS-MS. This combination of state-of-the-art techniques should provide more specific information with less sample cleanup than was previously possible.

V. DISCUSSION

A. LC-MS Interfacing

The subdivisions of the previous sections are not as clearly defined as would first appear, and any attempt to classify the various approaches to LC-MS inevitably results in overlap. For example, an atmospheric pressure ionization mass spectrometer could be used in conjunction with any of the LC-MS interfaces, and similarly so could CID. Also the more recent developments of the DLI LC-MS incorporate some method of nebulization of the liquid jet to increase the efficiency of ionization.


UV
(a)
(b)
65
92
108
156
311
0
2
4
6
8
10
12
minutes

It is obvious that an ideal method for the combination has not yet been developed, and it appears unlikely that one interface which will allow an unrestricted LC-MS coupling will be developed in the near future. However, the advantages of on-line LC-MS far outweight the disadvantages in the majority of cases. Both EI and CI mass spectra are available in many cases. There is also the potential for soft ionization techniques which could provide data on compounds otherwise too unstable for LC-MS analysis [125].

In order to accomplish sensitive on-line LC-MS, complete transfer of sample on-column and splitless transfer from column exit to mass spectrometer is required. Thus with all the LC-MS interfaces covered here, low eluent flow-rate is a considerable advantage. Recent advances in HPLC technology include the use of narrow-bore liquid chromatography columns (i.e., narrower than the conventional 4.6 mm i.d. columns) with low flow-rates. There are certain problems associated with the use of narrow-bore columns such as packing columns which would routinely have plate counts equivalent to those obtained with conventional columns. However, commercial microbore (1 mm i.d.) columns are available with plate counts of at least 40,000 plates per meter. Very high plate counts (above 100,000) can be obtained using open-tubular columns or catenation of microbore columns [100]. Unfortunately, these plate counts are produced after very long elution times (i.e., several hours). Thus, in order to achieve a practical sample analysis time of less than 30 min, it appears necessary to sacrifice plate count to some extent.

The DLI and moving-belt approaches to LC-MS interfacing have had the most success and consequently the most use. Although both approaches have been shown to work well, neither system can demonstrate any overwhelming advantage, except perhaps that DLI LC-MS interfaces are less complex and therefore cheaper, and simple "homemade" interfaces can be constructed. In fact, it would seem possible that any future major breakthrough in the field is more likely to come from the thermospray interface approach, since this appears to have great potential.

B. LC-MS Applications

A literature survey of the LC-MS papers published to date shows that although numerous papers have been published on LC-MS interfacing,

Figure 10 LC-MS-MS analysis of an extract of race horse urine containing sulfadimethoxine. LC conditions: column 25 cm × 4.6 mm PXS ODA; 1 mL min^{-1}, methanol:water, linear gradient 10:90 to 90:10 over 10 min. (a) UV chromatogram; (b) API SIM LC-MS-MS traces resulting from CID of *m/z* 311 $[M + 1]^+$ ion of sulfadimethoxine. (Reprinted with permission from Ref. 66. Copyright 1982 American Chemical Society.)

very few in fact illustrate practical examples in which LC-MS can be shown to solve real-life problems. The majority of the work has been carried out using "trivial" compounds such as hydrocarbon mixtures which could be easily analyzed by GC-MS. Although these samples are often being used to test out new interface developments, they do little to illustrate the realistic potential of the technique.

A few research groups have, however, published viable applications such as identification of steroid and pesticide metabolites in biological fluids, and drug assays. Hopefully this work will continue and will demonstrate the potential to others who might wish to try the technique.

VI. CONCLUSION

It can be seen that LC-MS, whether off-line or on-line, is a useful analytical tool and that a viable on-line system will always be preferred. On-line LC-MS allows chromatographic integrity to be retained and minimizes sample handling and operator time. An ideal LC-MS has not yet been developed, but those that are available can be used to produce worthwhile results. Hopefully, interest in LC-MS will continue to increase as more people realize that LC-MS can be routine and that it can provide useful information.

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7

DRUG DETERMINATION IN BIOLOGICAL FLUIDS BY LIQUID CHROMATOGRAPHY-FLUORESCENCE

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I. INTRODUCTION

It is well established that liquid chromatography suffers from the general detection problem: the lack of a single detector that is responsive, with adequate sensitivity, to all compounds. However, a universal detector is by definition unselective. A series of detectors, each selective to its various applications, represents the ultimate goal of drug analysis in complex matrices such as biological fluids.

Molecular luminescence is generally recognized as one of the most sensitive analytical techniques that can be performed with relatively low-cost instrumentation. Under optimum conditions, with ideal emitters, the attomole range is accessible. More frequently, femtomole and picomole sensitivities are obtained, but these are one or more orders of magnitude lower than the ultraviolet absorption measurements. The reason for this enhanced sensitivity is instrumental. A photomultiplier tube can best differentiate a small signal from a low background, rather than the difference between two large signals, as in absorption measurements.

Luminescence measurements are also selective. Not all compounds that absorb radiation will emit. Of course, this is a two-edged sword, since the technique will be limited to a narrower range of compounds. Chemists have addressed this problem with impressive creativity by designing derivatives that maintain the selectivity of luminescence detection, while still taking advantage of the sensitivity.

Fluorescence is only one of several luminescent phenomena that can be observed from molecules. Both phosphorescence and sensitized phosphorescence have been employed in liquid chromatographic detec-

tion, and these developing techniques may have a role in improving future analysis.

This chapter will review the fundamental concepts of molecular luminescence, the instrumental techniques for measurements, pre- and postcolumn derivatization, and the latest concepts in LC luminescence detection. This latter section will include chemiluminescence (CL) and room-temperature phosphorescence (RTP) detection in LC. The variety of luminescence schemes and derivatization procedures provides the means for obtaining sensitive and selective detection in LC.

The literature survey from which the applications were extracted is comprehensive though not exhaustive. Particular attention is given to literature from 1979 to 1983, since assays for the most important drugs tend to be improved and reappear in the literature. Multiple citations for many drugs were found. Generally, only the most recent reference is included, unless earlier papers reported low limits of detection, unusual sample preparation, particularly attractive chromatography, or multiple applications. Papers that did not report limits of detection are not included in the applications tables (Tables 2-4) but may be cited in the text. Citations for promising, as well as established, drugs are included in this survey.

Limits of detection, as reported by some researchers, may not be useful for evaluating the ultimate detectability of a method. Frequently, they are reported in units of concentration, for example, 10 ng/mL, but this number is dependent on the sample size and sample preparation. Often, substantially lower limits of detection can be obtained by modifying the sample preparation steps. This is usually the case for procedures dedicated to therapeutic drug monitoring, where the assay is designed for a limited working range. It would be useful if authors would report their limits of detection in two forms: (a) units of concentration, and (b) the mass that can be detected, that is, the absolute number of nanograms. As a general guide to detectability, the limits of detection, in units reported in the individual papers, are included in the applications tables.

II. BASIC CONCEPTS

Luminescence is the general term which describes the emission of radiation that occurs after a molecule has absorbed energy and is elevated to an excited state. This phenomenon is one of several mechanisms that an excited species can undergo to rid itself of excess energy. Some of these photophysical processes are illustrated in Fig. 1.

Absorption of radiation occurs when the energy of the incident light corresponds to the energy differential between a ground and an excited state. The energy of that transition and its relationship

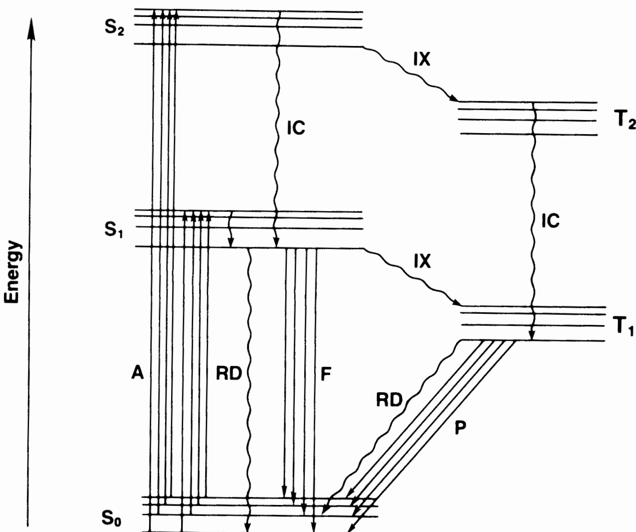

Figure 1 Simplified energy level diagram for an aromatic molecule. Key: S_0, ground state; S_1, S_2, excited singlet states; T_1, T_2, triplet states; A, absorption; F, fluorescence; P, phosphorescence; RD, radiationless deactivation; IC, internal conversion; IX, intersystem crossing. Solid lines indicate a radiative process, wavy lines indicate a radiationless process.

to the frequency and wavelength of the exciting light is given by the fundamental equation:

$$E = h\nu = \frac{hc}{\lambda} \qquad (1)$$

where

E = energy

h = Planck's constant

ν = frequency of light

c = speed of light

λ = wavelength of light

Upon absorption of a photon, a molecule is excited to one of the singlet states: $S_1, S_2, \ldots, S_n$, depending on the energy of the transition. The entire absorption process occurs in about 10^{-15} sec. Emission is seldom observed from the higher singlet states, as $S_n - S_{n-1}$ internal conversion proceeds until the absorbed photon, now an electron, resides in S_1.

All molecular electronic states have vibrational and rotational energy levels associated with it. Rotational transitions are seldom observed in fluid solution in the ultraviolet/visible spectral region, but vibrational transition have profound effects on the molecular spectrum. Figure 2 shows a potential energy diagram of ground and excited states with their associated vibrational energy levels.

Immediately after absorption, the electron is in one of the higher vibrational levels of a singlet excited state. Emission does not occur from that state, but the energy first decays to the zero vibrational level through internal conversion. Since the time for these photophysical processes is negligible compared to nuclear motion, the most probable (intense) transition involves no change in the position of the ground and the excited states. Thus, the Franck-Condon principle provides a rationale for the observed intensity of certain spectral bands, and internal conversion accounts for emission occurring at longer wavelengths than absorption. Because of internal conversion, the wavelengths of emission are constant regardless of the excitation wavelength employed.

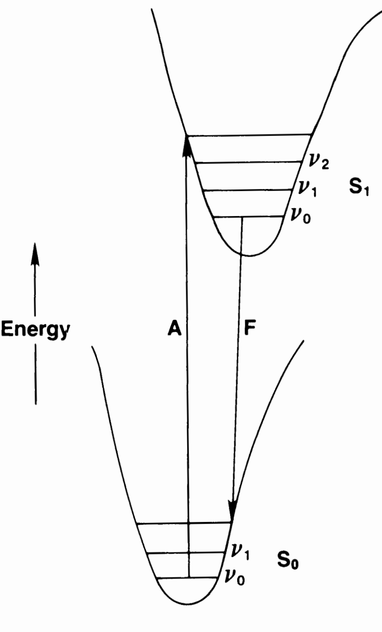

Figure 2 Simplified potential energy diagram for an aromatic molecule showing the various vibrational states associated with the ground and first singlet excited state. The absorption and fluorescence processes indicated are those predicted by the Franck-Condon principle.

Following internal conversion, there are three major processes which can occur, all of which are competing:

1. The molecule can return to the ground state via radiationless decay. This occurs in a time frame of 10^{-13}-10^{-11} sec and is due to vibrational and collisional mechanisms.

2. Another radiationless pathway is intersystem crossing to the triplet state. This process is spin forbidden by quantum mechanical selection rules, but nevertheless is an important deactivation mechanism. The transition from the triplet state to the ground state is also spin forbidden and has a lifetime which can approach 10 sec. Because of this long life, the triplet state is vulnerable to numerous quenching mechanisms that result in radiationless decay to the ground state. When emission does occur, it is known as phosphorescence. Since the triplet state is of lower energy than the singlet state, emission occurs at even longer wavelengths than fluorescence. Phosphorescene is seldom observed in fluid solution at room temperature, unless special conditions are employed. The excitation, fluorescence, and phosphorescence spectra of propranolol are shown in Fig. 3. These spectra

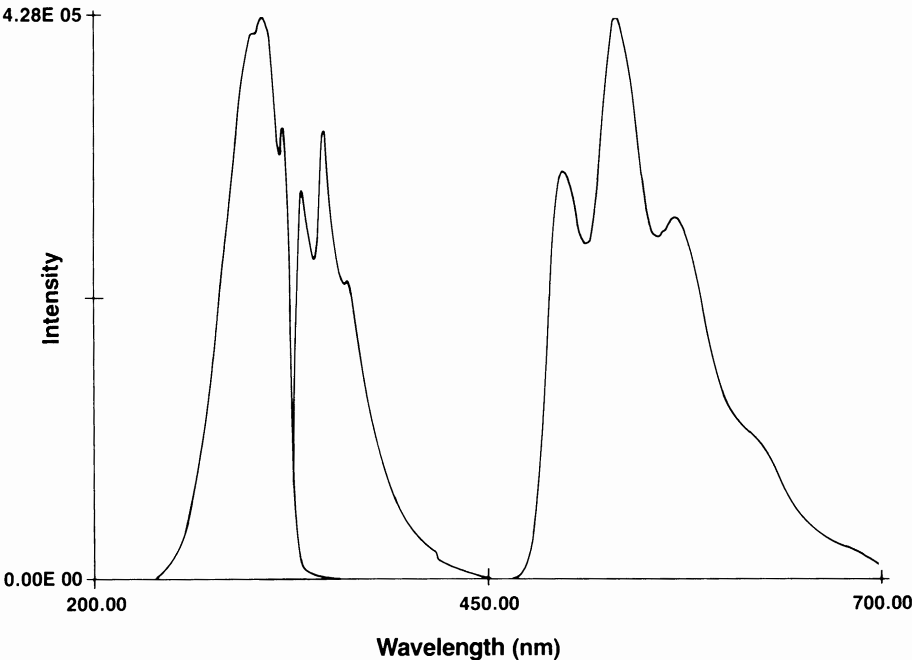

Figure 3 Excitation, fluorescence, and phosphorescence spectra of propranolol in micellar media. Propranolol, 10^{-4}M in 0.1 M sodium/thallium dodecyl sulfate (70%/30%).

were obtained by a technique called micelle-stabilized, room-temperature phosphorescence, which will be discussed later in this chapter.

3. The third, and most analytically useful, event that can occur is fluorescence. Fluorescence, like phosphorescence involves return to the ground state with emission of a photon. However, fluorescence occurs within 10^{-9}-10^{-7} sec after excitation, so it is less subject to the quenching mechanisms that affect phosphorescence. Rigid molecules, such as polycyclic aromatic hydrocarbons, which have few degrees of freedom, are generally strong fluorescers. In contrast, aliphatic molecules, which have many degrees of freedom and closely spaced energy levels, relax by internal conversion without significant emission.

The intensity of fluorescence emission is the product of a series of terms that relate to basic molecular properties and instrumental parameters. The general intensity equation (2) takes into account some, but not all, of these parameters:

$$I_f = I_0 \Phi \text{ abc k} \qquad (2)$$

where

I_f = intensity of fluorescence

I_0 = intensity of excitation source

Φ = quantum yield

a = absorptivity

b = path length

c = concentration

k = instrumental constant

Since k depends upon both wavelength and the instrument's optical efficiency, fluorescence data taken on different instruments may not be reproducible unless correction factors are applied. The fluorescence intensity is proportional to solute concentration only in dilute solution where phenomena such as self-absorption, excimer formation, and inner-filter effects are negligible.

The quantum yield, Φ, is a fundamental molecular parameter which describes the efficiency with which a compound converts absorbed light to fluorescence intensity (3):

$$\Phi_f = \frac{\text{\# photons emitted}}{\text{\# photons absorbed}} \qquad (3)$$

The quantum yields for aromatic molecules can approach zero or unity, with most falling somewhere in between. It can be influenced by the environment experienced by the solute (e.g., polarity, pH, concentration) and by the presence or absence of quenchers (e.g., heavy atoms and oxygen).

III. INSTRUMENTATION

The wide variety of fluorescence instrumentation available today can present difficulties for the analyst in replicating previously reported work. The problem of fluorescence is the lack of an absolute scale of luminescence intensity. This problem is compounded by the diversity of light sources, optics, monochromators, filters, and photomultiplier tubes (PMT), that comprise the fluorescence detector. For comparison, no such problems are found with absorption detectors. Fundamental measurements such as wavelength maxima and molar absorptivity are easily replicated on an inter-instrument and inter-laboratory basis because the scale for absorption measurements is rigorously defined. Either a sample has zero transmittance (100% absorbance), 100% transmittance (zero absorbance), or falls somewhere in-between.

Sophisticated fluorescence instruments utilize correction devices to account for both light-source intensity changes, monochromator efficiencies, and photomultiplier response variations at different wavelengths. Corrected spectra are generally repeatable, but instrumentation of this caliber is seldom used for liquid chromatographic detection.

The basic components of an LC fluorescence detector are shown in Fig. 4. Most instruments measure emission at right angles from illumination, but this is not universal. Flow cells are generally in the range of 5-20 μL.

There are three configurations of importance for these detectors, distinguished by the monochromator or filter arrangements that are used for wavelength selection. These are filter-filter, monochromator-monochromator, or monochromator-filter. Filter instruments or fluoro-

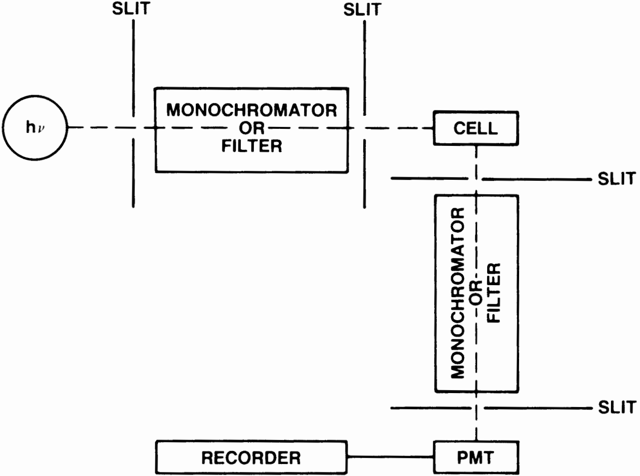

Figure 4 Simplified schematic diagram of a typical fluorometer or spectrofluorometer.

meters tend to be most sensitive if optimum lamp/filter combinations can be selected, but this is often not possible. Dual-monochromator instruments or spectrofluorometers are the most selective since relatively narrow band-passes can be chosen for both excitation and emission. If equipped with scanning drives, this instrument can provide complete excitation and emission spectra. However, unless the instrument band-pass is opened, spectrofluorometers are generally less sensitive than fluorometers. The most widely used LC detector, the Kratos FS 970 has the monochromator-filter configuration. This arrangement combines features of the other two instruemnts and offers a reasonable comprimise between selectivity and sensitivity.

The light sources used in fluorescence detectors are either line or continuum lamps. Line sources are generally used in fluorometers. Mercury, zinc, or cadmium lamps emit intense spectral lines at discrete wavelengths. If a compound's optimum absorption band matches an intense line emission, the method can be very sensitive.

Several types of continuum sources are used in LC spectrofluorometric detectors. The most common is the xenon arc, whose emission profile is shown in Fig. 5. Xenon arc emission is most intense in the near ultraviolet and visible region but falls off sharply in the ultraviolet wavelength range.

A second and less common source is the deuterium lamp. These lamps are generally less powerful but more stable than xenon arcs. Source stability is important because background is the limiting factor in detectability. This instability manifests itself as noise due to oscillation of background emission. As will be shown later, chemilumines-

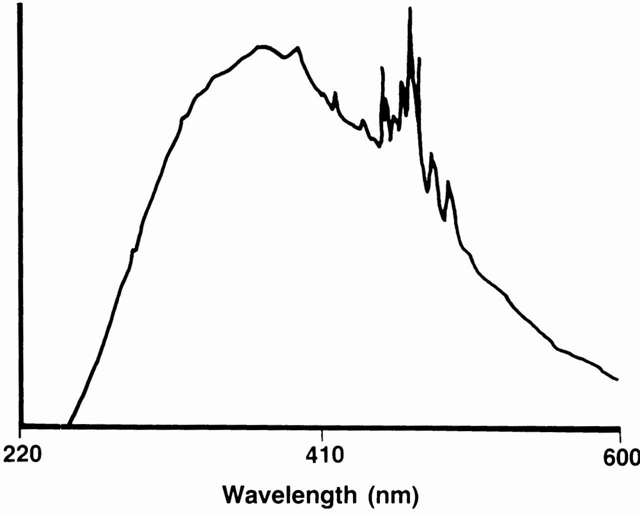

Figure 5 Relative energy output of a xenon arc excitation source. The spectrum is not corrected for grating or photomultiplier tube efficiency.

cence can offer impressive sensitivity since the fluorophore is excited chemically rather than photochemically.

The spectral profile of the deuterium lamp is shown in Fig. 6. These lamps have intense emission in the ultraviolet region of the spectrum with a maximum at 230 nm. Very different excitation spectra are obtained with the deuterium lamp compared to the xenon arc. This is due to the different spectral output of the individual source. An example of these differences is shown in Fig. 7.

Entirely different absorption bands are excited with each source. Because of the xenon arc's intense near-UV emission, the less intense absorption band at 360 nm is the optimal excitation wavelength. In contrast, the optimal excitation wavelength with the deuterium lamp is 240 nm, which falls near the absorption maximum for this compound. Generally, *the greatest analytical sensitivity will be obtained whenever the maximum lamp emission corresponds to a solute's optimal absorption band*. For compounds that absorb in the UV, the deuterium excitation spectra can be similar to the absorption spectra. The excitation maxima for several compounds, with both xenon and deuterium lamps, are shown in Table 1. For these cases, the excitation maxima with deuterium fall near the absorption maxima. However, these data show that the xenon arc is more selective because of the excitation wavelength discrimination that is found with that source.

Low-UV excitation has other advantages that relate to chemical and instrumental features. For example, Raman bands are due to inelastic scattering of the primary exciting radiation. In the low UV, this shift is usually 20-30 nm with solvents such as water or methanol. Recalling that the wavelengths of solute emission are independent of the wavelength of excitation, the Raman scattering is easily removed with a cutoff filter. With near UV excitation, the Raman band may result in elevation of background, particularly for compounds with small Stokes shifts. When using xenon arcs or tungsten-halide lamps

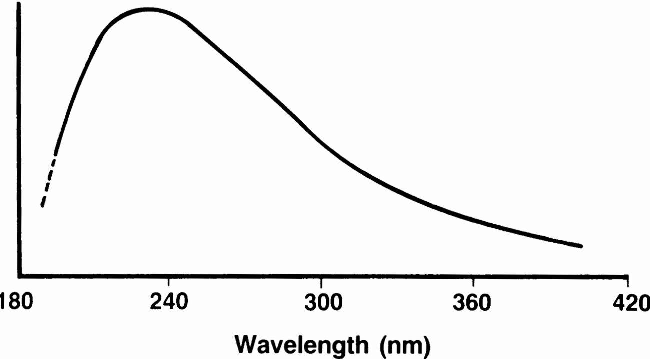

Figure 6 Relative energy output of a deuterium lamp excitation source.

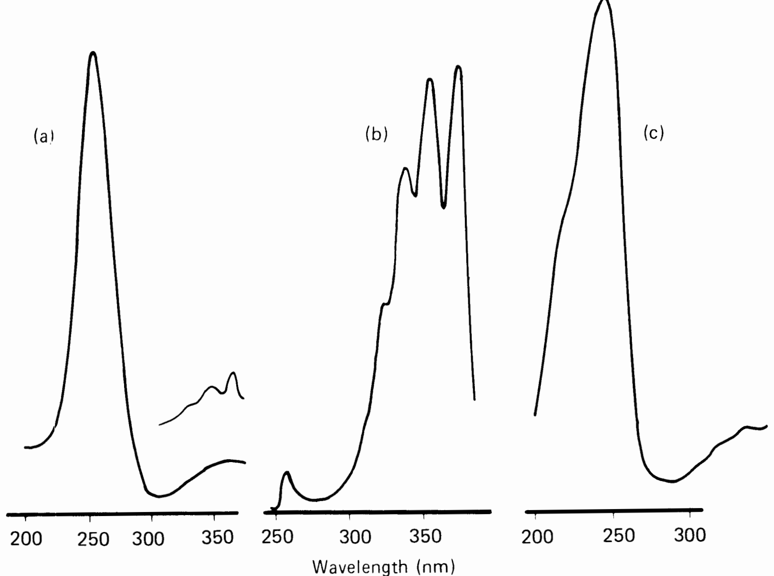

Figure 7 (a) Absorption spectrum; (b) xenon arc excitation spectrum; and (c) deuterium lamp excitation spectrum for anthracene.

(another common source), care should be taken to screen the photomultiplier tube from the Raman band, or substantial losses in sensitivity may be encountered.

When using a grating monochromator for selection of emission wavelengths, another potential contribution to background is second-order radiation. All gratings pass higher orders of radiation that are mutliples of the selected wavelength. These higher orders are less intense than the first order, and normally, only the second order is important.

Table 1 Optimum Excitation Wavelengths with Xenon and Deuterium Sources

	Wavelength (nm)	
Compound	Xenon	Deuterium
Naphthalene	285	231
Anthracene	365	240
Pyrene	333	236

This problem may be encountered when the emission wavelength is double the excitation wavelength. In this case, the emission monochromator is transparent to scattered excitation radiation, and as a result, the background signal is elevated. The solution is to adjust the excitation and/or emission monochromators to stay away from the second order. If this is unavoidable, a cutoff filter, which removes scattered excitation radiation, may be placed at the entrance of the emission monochromator. Minimizing some of these sources of background will result in optimizing the sensitivity of analyses. With these features considered, the major contribution to background is fluorescent impurities in the chromatographic mobile phase.

IV. APPLICATIONS OF LC-NATIVE FLUORESCENCE

The analyst is quite fortunate when a solute has significant native fluorescence. Sample preparation is usually simplified owing to the selectivity of the detector and the total material recovery requirements can often be relaxed due to the sensitivity. However, the power of this detector can give a false sense of security. Thorough sample preparation is usually rewarded by cleaner chromatograms and shortened chromatographic time.

The simplest procedures employed for the determination of drugs in plasma involve denaturation of plasma proteins with solvents such as acetonitrile, or precipitating agents like trichloroacetic acid. After centrifugation, as aliquot of the supernatant is injected directly into the LC. This method has been applied to bumetanide [1], griseofulvin [2], and thiabendazole [3]. The disadvantage of this technique is that endogeneous plasma components appear in the early portion of the chromatogram. This requires adjustment of the separation, and the chromatographic time is often longer than optimum. There is always the danger of late-eluting peaks, interfering in subsequent chromatograms. Early-eluting metabolites may be obscured by other early eluting peaks.

In Chiou and co-workers' method for griseofulvin [2], as little as 25 μL of plasma was deproteinized with 2.5 volumes of acetonitrile. The mixture was centrifuged, and 50 μL was injected into the LC. The mobile phase, 50% acetonitrile, was used at a flow-rate of 2.0 mL/min with a μBondapack C_{18} column. Under these conditions, the drug eluted in 3.8 min. The Kratos FS 970 fluorescence detector was set with an excitation wavelength at 260 nm, and KV 389 interference filter selected the emission wavelengths. A limit of detection of 50 ng/mL was obtained. This group has employed similar sample preparation schemes for the determination of creatinine, aspirin, salicylic acid, theophylline, procainamide, and *N*-acetylprocainamide in plasma. They found that 2.5:1 ratio of acetonitrile to plasma was important to insure complete protein precipitation and thus prolong column life.

Although appropriate for its intended use, the above method would not be useful for ultratrace measurements of the drug for two reasons. First, increasing the detector sensitivity, if possible, would result in amplifying the early-eluting peaks, which would either place the PMT into shutdown, or simply mask the eluting drug. And second, without the ability to preconcentrate the sample, high-sensitivity levels would be unobtainable.

Solvent extraction can mediate both of these problems by removing fluorescent, water-soluble impurities and allowing the extract to be concentrated by evaporation. Procedures employing solvent extraction, evaporation to dryness, followed by reconstitution into a small volume of mobile phase are often used for sample preparation. Frequently, the plasma sample is buffered to an appropriate pH to provide for selectivity of extraction and maximal recovery. An internal standard is introduced prior to the extraction. If sensitivity is adequate, the evaporation step can be eliminated. When using reversed-phase separations, small amounts of organic solvents can be injected with only small losses in resolution, provided the mobile phase is sufficiently strong to solubilize the bolus.

Examples utilizing single-solvent extractions include glyburide [4], furosemide [5], labetalol [6], alizapride [7], and naproxen [8]. The method for naproxen by van Loenhout et al. [8] compared sensitivity and precision obtained with both UV and fluorescence detection. In their method, 0.5 mL plasma, an internal standard (diflunisal for UV; ethylnaproxen for fluorescence), and some hydrochloric acid were extracted with 5 mL of ether:hexane (1:1). The organic layer was evaporated to dryness and the residue reconstituted in 1 mL of mobile phase. A similar procedure was used for urine samples except that acetic acid was used for acidification. This was done because desmethylnaproxen is unstable in HCl. This metabolite is never found in plasma, so HCl was employed for those samples because of its superior protein-precipitating power. Chromatography was performed on a Lichrosorb RP 8 column with a mobile phase of 50% methanol in pH 6.5 citrate buffer. A 40% methanolic mobile phase was used for the urine samples. The chromatogram of the urine extract is shown in Fig. 8.

Fluorescence detection was performed with a Perkin-Elmer 3000, set at an excitation wavelength of 235 nm (slit width, 10 nm) and an emission wavelength of 350 nm (slit width, 5 nm). The UV detector was a fixed-wavelength (254 nm) instrument.

For fluorescence detection, a limit of detection (LOD) of 100 ng/mL with 0.5-mL samples was found, with corresponding relative standard deviation (RSD) of 3.9%. For UV detection, the LOD was 1000 ng/mL with an RSD of 2.4% at the 5 μg/mL level. The LODs for either of these assays could probably be improved, if necessary, by a greater sample concentration step or by employing a deuterium excitation source.

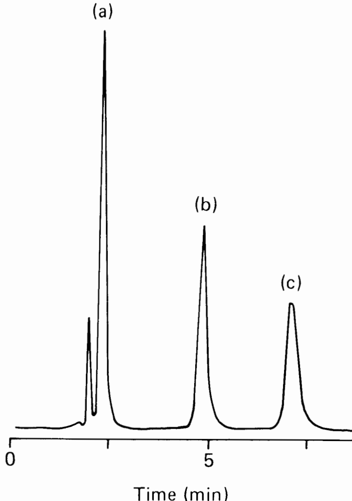

Figure 8 Chromatogram of 6-O-desmethylnaproxen (a), naproxen, (b), and ethoxynaproxen (c) after extraction from urine. Column, Lichrosorb RP 8; mobile phase, methanol/citrate buffer (pH 6.5), 50%/50%; flow-rate, 1 mL/min.; excitation wavelength, 235 nm (slit width, 10 nm); emission wavelength, 350 nm (slit width, 5 nm). (Reprinted with permission from Ref. 8.)

Sample preparation with multiple extractions is most often used for drug assays in biological fluids. Since most drugs are ionizable, back extractions can be applied for both cleanup and sample concentration. These methods are generally used when very low LODs are required. Beta blockers [9] such as propranolol, metoprolol, soltolol, acebutolol, and atenolol are prepared via back extractions. Ergot alkaloids [10] also may be cleaned up with back extraction, and multiple extractions are employed for LSD [11]. These methods all have low to sub-nanogram per milliliter LODs and demonstrate the capability of good sample preparation combined with fluorescence detection.

Lefebvre and co-workers [9] explored the ideal extraction, chromatographic, and detection conditions for a series of fluorescent and nonfluorescent beta blocker. The nonfluorescent compounds included in their study were timolol, pindolol, and oxprenolol.

The authors found that alkalization of plasma with sodium hydroxide to pH 9-10, extraction with chloroform/pentanol (60/20), followed by back extraction into 0.1 N H_2SO_4 gave optimum results for all of the beta blockers. A μBondapak C_{18} column was used for the separations, and mobile phases of methanol/acetic acid/water from 28/1/71 to 50/1/49, all prepared by volume, were used depending on the specific compound.

The fluorescence detector, a Kratos FS 970, was set at excitation wavelengths from 215 to 235 nm, and no filter was used for emission except for acebutolol, where a 389-nm cutoff filter was utilized. The LODs ranged from 0.5 ng/mL for propranolol to 10 ng/mL for acebutolol and soltolol. Of the UV-detected compounds, timolol had the highest LOD of 40 ng/mL. Typical chromatograms are shown in Fig. 9.

The preceding examples have illustrated some of the features and problems found in assays of this type. All forms of sample preparation were not included, for example, solid-phase extraction. It is quite clear that as limits of detection decrease, the demands on optimization of all parameters becomes more important. As a general guide to the scope of native fluorescence in LC detection, an applications list is provided in Table 2.

V. PRECOLUMN DERIVATIZATION

Derivatization techniques in liquid chromatography have been receiving increasing attention in recent years because of the potential of enhancing sensitivity and selectivity for molecules that lack native fluorescence or have weak chromophores. A comprehensive chapter on the subject was published in 1981 [30].

In liquid chromatography, derivatization can take place either pre- or postcolumn, and each method has inherent advantages and shortcomings. Many of the reactions that are used postcolumn are also applicable for precolumn work, but the reverse is generally not true. A fundamental requirement for postcolumn chemistries is that the reagents contribute little to the background signal. For precolumn techniques, the reagent or tag is usually fluorophoric or chromophoric and the unreacted tag is removed by extraction or separated chromatographically from the derivatized solute. This is not possible in postcolumn techniques.

This section will review some important precolumn derivatizing agents. Reactions generally used for postcolumn systems will be discussed in the next section, though some of these are also used in precolumn techniques. The applications list (Table 3) provides references for these and other derivatization procedures not described in this section.

The role of derivatization in LC is primarily to enhance detection. Unlike in gas chromatography, derivatization is seldom used to improve the separation. In LC, this feature generally is unimportant because of the wide variety of mobile-phase additives which permit adequate resolution of most mixtures.

The principal advantages of precolumn over postcolumn techniques are:

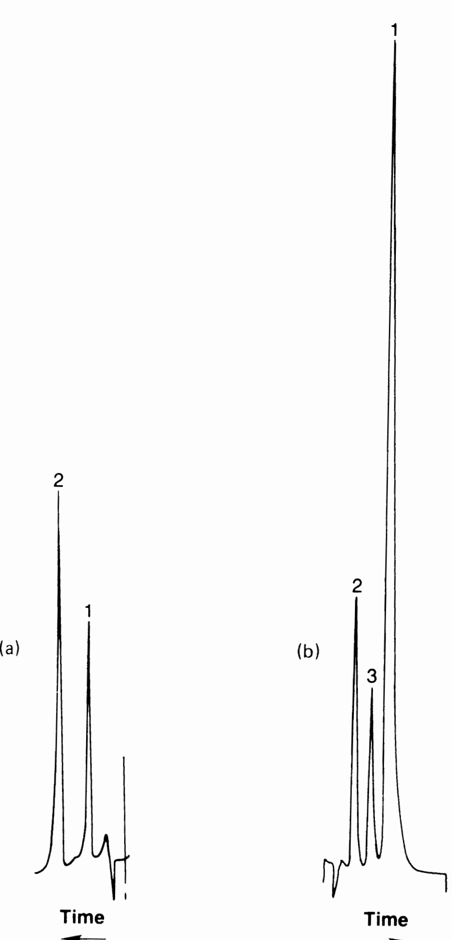

Figure 9 (a) Chromatogram of plasma samples spiked with 1 μg of metoprolol (2) and 1 μg of acebutolol metabolite (1). Column: μBondapak C_{18}. Mobile phase, 50/1/49, methanol/acetic acid/water; flowrate, 1.3 mL/min; excitation wavelength, 222 nm; retention time of metoprolol, 4.8 min. (b) Chromatogram of plasma samples spiked with 0.25 μg of propranolol (1) and 4-hydroxy propranolol (2), and internal standard (3). Mobile phase and flow as in (a); excitation wavelength, 215 nm; the retention time of propanolol is 8 min. (Reprinted with permission from Ref. 9.)

Table 2 Applications of LC-Native Fluorescence for the Determination of Drugs in Biological Fluids

Drug	LOD (ng/mL)	Reference
Acebutolol[a]	5	9
Alizapride	5	7
Atenolol	10	9
Bromo-lasalocid	100	13
Bumetanide	5	1
Carubicin	1	12
Citalopram[a]	1	14
Desipramine[a]	5	17
Dihydroergocristine	5	15
Dihydroergotamine	5	15
Doxorubicin	5	12
Ergotamine	0.1	10
Ergotaminine	0.2	10
Furosemide	100	5
Glyburide	10	4
Griseofulvin	50	2
Ibuprofen	1000	16
Imipramine[a]	5	17
Ketanserin	0.5	18
Labetalol	1	6
Lysergic acid (LSD)	0.5	11
Marcellomycin	5	12
Meptazinol	3	19
Methylergometrine	0.1	10
Metolazone	1	20
Metoprolol[a]	5	21
Naproxen[a]	0.1	8
Prazosin	1	22
Prenalterol	1	23
Propranolol	0.5	9
Protriptyline	5	17
Pyrimethamine	10	24
Quinidine	5	25
Salicylic acid[a]	300	26
Sotalol	10	9
Sulpiride	10	27
Thiabendazole[a]	1000	3
Verapamil	1	28
Viloxazine	25	29

[a]Metabolites reported.

Table 3 Applications of LC with Precolumn Derivatization for the Determination of Drugs in Biological Fluids

Drug	Chemistry	LOD (ng/mL)[b]	Reference
Amikacin	FDNM (UV)[c]	2,000	41
Aminocaproic acid	Fluorescamine	50,000	42
Amphetamines[a]	NQS[d]	2	40
Anabolic steroids	Dansylation	5ng	36
Antihistamines	Dealkylation	0.5 ng/20 μL	37
Barbiturates	Dansylation	2	31
Captopril	NPM[e]	150 pmol/mL	43
Endralazine[a]	Hydrolysis	1 pmol/mL	44
Enkephalins	Fluorescamine	2ng	45
Gentamicin	OPA[f]	500	46
Hydralazine	Hydrazone formation (UV)	5	47
Hydrocortisone	Dansylation	1	48
Indomethacin[a]	Deacylation	25	49
Methotrexate	Oxidation	10	50
Morphine	Dimerization	10	51
Neomycin	FDNB (UV)[c]	1	52
Perhexiline maleate	Danyslation	5	34
Phenylpropanolamine	OPA[f]	5	53
Pilocarpine	Quaternization (UV)	4	54
Prostaglandins	Coumarin	10 fmol	39
Steroids	Dansylation	60 pg	55
Tamoxifen[a]	Photolytic	1	56
Tetrabenazine[a]	Oxidation	0.1	57
Tocainide	Fluorescamine	100	58
Trimetazidine	Dansylation	1	32
Valproic acid	Phenacylation	10,000	38

[a]Metabolites reported.
[b]Or in units as specified.
[c]1-fluoro-2,4-dinitrobenzene.
[d]β-naphthaquinone-4-sulfonate.
[e]*N*-(1-pyrene)-maleimide.
[f]*o*-phthalaldehyde.

1. No special hardware is required.
2. Effects of band broadening on chromatographic resolution are not encountered.
3. Long reactions can be employed.
4. Aggressive reagents can be used.
5. Fluorophoric or chromophoric tagging procedures are possible since the excess reagent will be removed or separated chromatographically.

A. Dansylation

Dansyl chloride [1] and its more lipophilic analogues are used to derivatize

$N(CH_3)_2$

SO_2Cl

1

primarily and secondary amines and phenols with a fluorescent tag. The reagent has been used to determine barbiturates [31], trimetazidine [32], perhexiline maleate [33], and tocainide [34] in biological fluids. Frei and co-workers [35] employed dansylation to determine adrenaline, ephedrine, emetine, cephaeline, and morphine in drug dosage forms. The reaction takes place under mild alkaline conditions at elevated temperature and is usually complete in 20-40 min. Rhys-Williams et al. [36] derivatized anabolic agents such as estriol, estrone, zeranol, HES, and DES. Dansyl hydrazine is useful in labeling keto groups with a fluorescent tag [55]. Limits of detection are generally in the picogram range for most dansyl derivatives.

B. Dealkylation

The lack of reactivity of the tertiary amine group has, until recently, prevented useful derivatization procedures in LC. A scheme by Gubitz and co-workers [37] uses 2-naphthyl chloroformate (NCF) [2] in the following reaction:

$$NR_3 + \text{C}_{10}\text{H}_7\text{–O–C(=O)–Cl}\ (\underline{2}) \longrightarrow \text{C}_{10}\text{H}_7\text{–O–C(=O)–NR}_2 + RCl \tag{4}$$

Between 10 pmol and 5 nmol of free base is heated with a 10-fold molar excess of NCF and 10 mg potassium carbonate (catayst) in 100 μL dry benzene at 100°C for 1 hr in a sealed vial. The excess reagent is consumed with 300 μL of saturated methanolic potassium hydroxide, and an aliquot of the benzene layer is injected into the LC. The derivatives are detected with a fluorometer. A separation of diphenylpyraline, diphenhydramine, and thenaldine is shown in Fig. 10. Limits of detection are in the low nanogram range, and the method appears suitable for biological matrices.

C. Esterification

Two important classes of tags are used to esterify carboxylic acids. For chromophore tagging, phenacyl or naphthacyl derivatives are common. For fluorophore derivatization, various coumarin compounds appear promising.

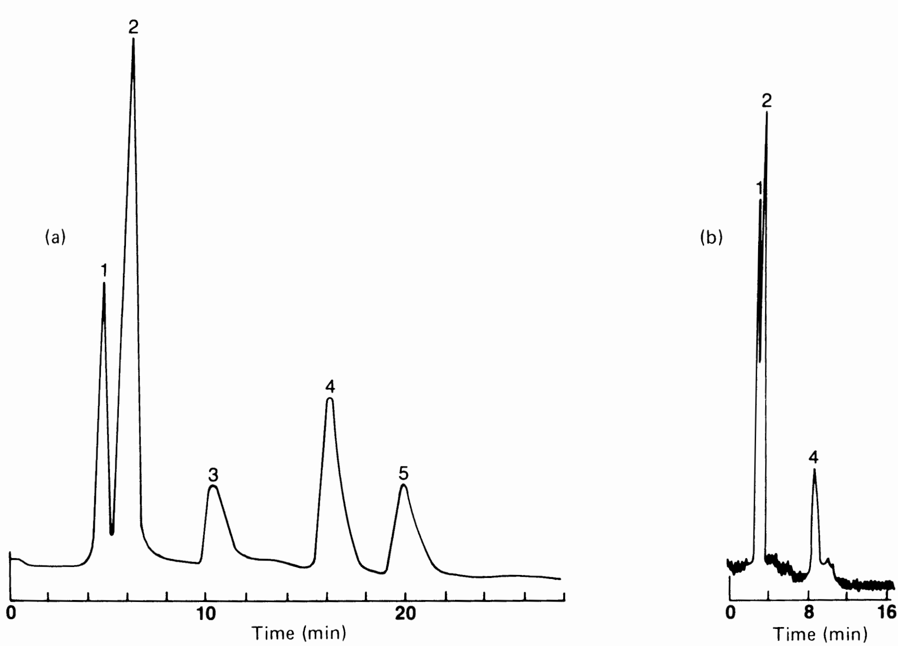

Figure 10 (a) Separation of antihistamine derivatives. Column, RP-18; mobile phase, 65/30/5, methanol/water/tetrahydrofuran; flow-rate, 2 mL/min; excitation wavelength, 275 nm; emission wavelength, 335 nm; 1,2 = decomposition products of reagent, 3 = diphenylpyraline, 4 = diphenhydramine, 5 = thenaldine. (b) Chromatogram of 500 pg diphenhydramine; conditions as in (a), except mobile phase, 80/20, methanol/water. (Reprinted with permission from Ref. 37.)

In Kline and co-workers' method for valproic acid [38], plasma extracts are heated with phenacyl bromide for 0.5 hr at 75°C. The reaction is crown ether-catalyzed and provides esters in better than 90% yield. The separation (Fig. 11) is performed on a C_{18} column with a 65% acetonitrile mobile phase. Detection is at 254 nm, and the LOD is less than 10 μg/mL under the conditions of the assay and sample preparation.

Prostaglandins can be analyzed in a similar fashion as described above; however, lower LODs are required. In the method of Tsuchiya and co-workers [39], 4-bromomethyl-7-acetoxycoumarin is used for crown ether-catalyzed esterification. Following the separation, the ester is hydrolyzed postcolumn, and the fluorescence of free coumarin measured. This combined pre- and postcolumn derivatization procedure provides for a fluorescence response that is uniform despite the nature of the prostaglandin derivative. Separations (Fig. 12) are well characterized and are complete in a little over 1 hr with gradient elution. Limits of detection are in the low femtomole range.

VI. POSTCOLUMN DERIVATIZATION

By employing derivatization in liquid chromatography, impressive improvements in both detector selectivity and sensitivity are generally observed. However, precolumn derivatization is not without disadvantages. The analyst must be aware of the potential for multiple products or artifact formation that can disturb the chromatogram. Chemical reactions must be carefully selected and conditions properly controlled to avoid imprecision in the analysis.

Postcolumn reaction detectors present an alternative and often superior solution to the general derivatization problem in liquid chromatography. The postcolumn approach can offer the analyst a means to optimize the separation and then manipulate the column effluent to enhance detectability. For example, barbiturates are best separated in an acidic mobile phase, but detectability can be improved substantially in an alkaline environment. By simple addition of a postcolumn alkalizing agent, the sensitivity was improved by a factor of twenty [59].

All postcolumn reactors are continuous-flow devices. This means the conditions under which reactions occur are consistent throughout the analysis. Selected reactions need not be driven to completion. The only criterion is that they be repeatable. Artifact formation is generally unimportant since no separation step subsequent to the reaction occurs.

Postcolumn reactions have certain inherent disadvantages. The reaction medium may be incompatible with the optimal mobile phase. Gradient elution may be difficult, if reaction kinetics change dramatically with mobile-phase composition. Very long reactions can result

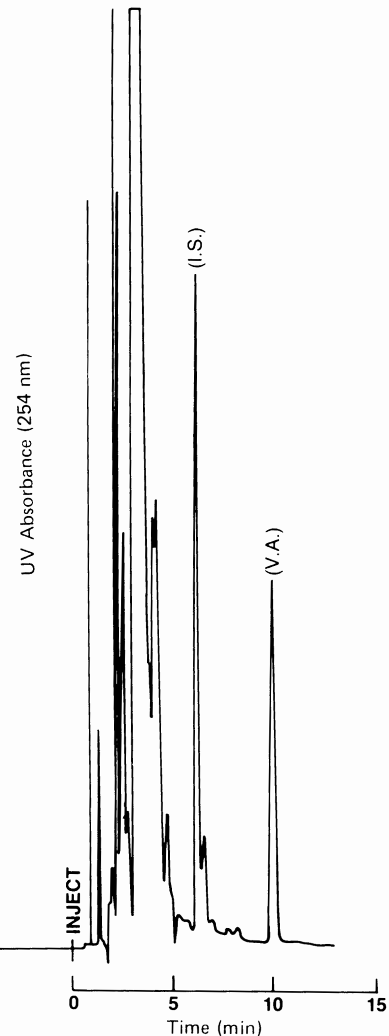

Figure 11 Chromatogram of a patient serum sample. Column, C_{18}; mobile phase, 65% acetonitrile; detection, 254 nm; internal standard, cyclohexane carboxylic acid; V.A. = valproic acid. (Reprinted with permission from Ref. 38.)

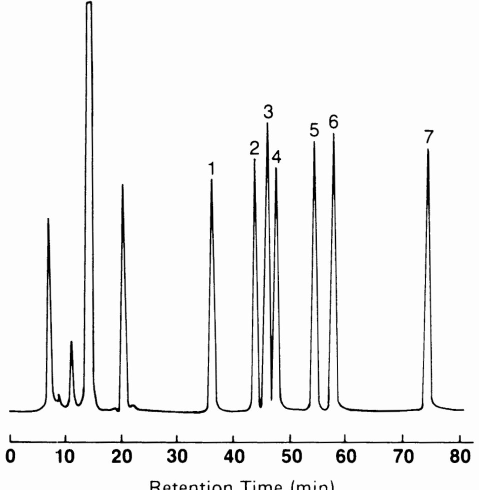

Figure 12 Separation of acetoxycoumarin prostaglandin derivatives. Column, RP-18; mobile phase, concave gradient from 30 to 60% acetonitrile. Excitation wavelength, 365 nm; emission wavelength, 460 nm. Peaks: 1 = 6-keto-PGF_1, 2 = PGF_2, 3 = PGE_2, 4 = PGD_2, 5 = PGB_2, 6 = 2-chlorothioxanthone (internal standard), 7 = arachidonic acid. (Reprinted with permission from Ref. 39.)

in significant band broadening, and the reagents must not contribute appreciably to detector background.

Growing interest in this field is evidenced by the number of review articles [60-63] and book chapters [64] that have appeared in recent years. Perhaps the most far-reaching advance in postcolumn chemistry is the generation of chemiluminescence as a means of detecting fluorophores [65-67]. Included in this section will be a description of the tyes of reactors used, some of the most common and versatile chemistries, and a list of applications (Table 4).

A. Reactor Types

The ideal postcolumn reactor should be designed to permit chemical reactions to be performed reproducibly, carried far enough to completion for sensitivity, and yet, introduce little extracolumn band-broadening. Because of diffusion processes that occur in fluid solution, all of various designs introduce some measure of band broadening. This is largely due to laminar flow and the interaction of the fluid stream with the walls of the reactor tubing. The goals of the reactor design are to minimize laminar flow by physical barriers or by encouraging secondary flow patterns in the reactor stream.

Table 4 Applications of Postcolumn Reactions for the Determination of Drugs in Biological Fluids

Drug	Reactor	Chemistry	LOD (ng/mL)[a]	Reference
Amoxicillin[b]	Tubular	Fluorescamine	2500	80
Barbiturates	Tubular	Alkalization	10 ng	59
Cannabinol	Tubular	Photolytic	1 ng	88
Cefatrizine	Bed	Fluorescamine	100	81
Clomiphene	Tubular	Photolytic	60 pg	84
Clobazam[b]	Tubular	Photolytic	70 pg	85
Digitalis glycosides	Segmented	Hydrochloric acid	500 pg	90
Erythromycin	Segmented	Extraction	50	91
Indomethacin	Tubular	Hydrolysis	1.5	92
Kanamycin	Tubular	OPA	20 ng	93
Morphine	Tubular	Dimerization	10 ng	94
Quinidine	Tubular	Acidification	50	75
Reserpine	Segmented	Nitrous acid	200 pg	95
Tamoxifen[b]	Tubular	Photolytic	200 pg	87
Thioridazine[b]	Tubular	Oxidation	3 ng	97
Warfarin[b]	Tubular	Alkalization	180 pg	76

[a]Unless other units specified.
[b]Reported metabolites.

1. Tubular Reactors

The simplest postcolumn reactor is the open-tubular system. The postcolumn reagents are pumped to the eluate stream, mixed in a low-volume tee, and reacted in a coil of appropriate length and diameter. These systems were studied by Deelder and co-workers [68]. From their work and that of others, the peak variance could be described by:

$$\sigma^2 = \frac{k d_t^2 t_v}{96 D_m} \tag{5}$$

where

σ^2 = additional peak variance

k = mixing constant

d_t = inner diameter

t_v = residence time

D_m = molecular diffusion coefficient

This means that minimal band broadening will be found for short reactions in very narrow tubes. The tubing diameter is generally required to be greater than 0.010 inch, or excessive tubing lengths would be required and severe pressure drops would be encountered, even for relatively short reactions. The diffusion coefficient, D_m, can be raised by increasing the temperature. The mixing constant, k, adjusts for the reactor configuration. For straight-line, open-tubular reactors, k = 1, but coiling the tubing results in a reduction of k, due to secondary flow patterns from radial forces.

The variance can be further reduced by using deformed tubing. This open-tubular reactor creates substantial side-flow due to the complex geometric features of the tubing. Though not theoretically well defined, these reactors have shown impressive performance with regard to their band broading characteristics [69,70]. These devices are commercially available from Kratos.

2. *Bed Reactors*

A bed reactor can be considered a packed chromatographic column that operates under zero retention characteristics. The semiempirical variance equation described by Hilby [71] (from Ref. [64]) suggests that band broadening can be minimized by using columns packed with very small particles, such as, glass beads. As in chromatography, both particle size and column length are limited by the pressure drop across the column. The bed reactor is particularily useful when the packing contributes to the reaction, as in Studebaker's example for the measurement of proteolytic enzyme activity [72]. Immobilized enzyme solid phase reactors are expected to play a significant role in many post-column chemistries.

3. *Segmented Stream Reactors*

Dispersion in open-tubular reactors can be greatly reduced by segmenting the flowing stream with uniformly spaced air bubbles or with an immiscible solvent. These bubbles create a physical barrier between mobile-phase segments which limits the diffusion between segments. The barrier is imperfect since leakage, due to wetting of the tubing walls, causes mixing with the next segment. A semiempirical equation describing band broadening in these reactors was recently developed by Snyder [73].

Virtually all of these reactors use Technicon AutoAnalyzer technology. Reagents are pumped with a peristaltic pump that employs flexible pump tubes of various materials depending on the solvents utilized. Upon its exit from the column, the mobile phase is generally segmented with air prior to introduction of chemical reagents. The multichannel nature of the peristaltic pump allows rather complicated chemistries to be performed by simply adding appropriate pump tubes and interconnecting them to the analytical manifolds. One of the dis-

advantages of this reactor is the changing flow characteristics of the pump tubes as they age.

The peak variance is generally minimized by careful manifold construction, high bubble frequency, short path lengths, high flow-rates, and a narrow inner diameter. Bubble frequencies of 1-2/sec and tubing diameter of greater than 1 mm are generally used. The flow-rates and tubing length are defined by the residence time required for the particular chemistry. Additional dispersion occurs in the debubbling step. This can be minimized by careful selection of minimal dead-volume fittings or by electronic bubble gating, that is, passing the bubble through the flow cell with computer reconstruction of the analytical signal [74].

B. Postcolumn Chemistry

While the reactor design represents the hardware of the system, the chemistry is the software. A wide variety of both chemistries and detectors are utilized in postcolumn reactions, but this section will concentrate on fluorescence methods.

The chemistry can be very simple, for example, a simple pH change, as in the analysis of barbiturates [59], quinidine [75], or warfarin [76]. These analyses permit the selection of the pH for both separation and detection. A simple open-tubular reactor with a short path length is sufficient since the reagents need only be mixed. Chemical reactions such as the *o*-phthalaldehyde (OPA) chemistry are more common. Light is also used as a postcolumn reagent by promoting photolytic reactions and fluorophore generation. Solvent extractions can also be performed, particularly with segmented stream technology. The newest and perhaps most exciting postcolumn technique involves chemiluminescence.

1. OPA (O-Phthaldehyde) and Fluorescamine

OPA [3] reacts with primary amines in alkaline media in the presence of a reducing agent like 2-mercaptoethanol. The reaction (6) involes ring

$$\underset{\underline{3}}{C_6H_4(CHO)_2} + RNH_2 + R'SH \longrightarrow \text{1-(SR')-2-R-isoindole} \qquad (6)$$

closure, and neither of the reagents is fluorescent. Excitation of the fluorophore is accomplished at 230 nm with a deuterium lamp or at 340 nm with the xenon arc. The emission maximum of the fluorophore is at 445 nm but may vary slightly, depending upon the particular primary amine. This reaction was first used for amino acids and was

later applied to aminoglycosides [77] and β-lactam antibiotics after alkaline hydrolysis [78]. Limits of detection are usually in the low nanogram range. Since the reaction occurs relatively rapidly at room temeprature, most reactor types are suitable, but the open-tubular is the simplest. This reaction is also used for precolumn derivatization [46,53], but is more suitable for postcolumn since the derivatives can be unstable. OPA will also react with peptides having an α-amino group. For the determination of secondary amines, an oxidative decarboxylation can be performed with hypochlorite followed by addition of the OPA [79]. Another pump is required, and the sensitivity is less than found with OPA alone, with primary amines. Spectinomycin, a secondary aminoglycoside, has been determined using this approach [96].

As with OPA, fluorescamine reacts with primary amines. Applications with amoxicillin [80] and cefatrizine [81] have been reported with low nanogram LODs. A two-pump reactor is required for alkalization and addition of fluorescamine. Unlike OPA, fluorescamine reagent is unstable at the reaction pH. However, fluorescamine can be used for detection of most peptides and proteins. The selection of either OPA or fluorescamine is based largely on experience, though OPA is generally the preferred reactant. The fluorescamine reaction may not be used with secondary amines, even after hypochlorite treatment.

2. Extraction Detectors

Extraction detectors permit the use of fluorescent reagents in postcolumn reactors, provided the unreacted reagent can be separated and does not contribute to background. This chemistry can be used advantageously to determine, for example, nonfluorescent tertiary amine drugs such as chlorpheniramine [82]. A flow diagram for one such system is shown in Fig. 13. In this case, an ion-pairing agent, dimethoxyanthracene sulfonate (DAS) solution, is air segmented and mixed with the mobile phase. An organic solvent is added, and an extraction is performed in a series of coils. The air and aqueous portion of the mixture are separated and the organic phase pulled through the fluorometer. The ion pair, formed only when an interactive solute elutes, is preferentially extracted into the organic phase. The DAS, being water-soluble, is discarded to waste. A disadvantage of this method is the restriction placed on the mobile phase. If modifiers much higher than 25% organic are present, the background becomes unacceptably high due to solubilization of free DAS.

Extraction detectors are somewhat more difficult to use than other reactors, but thier impressive sensitivity and selectivity can make them worthwhile. This is best illustrated by the chromatograms shown in Fig. 14 for both ultraviolet and extraction detection for HA, the major metabolite of the herbicide, atrazine. Though the chromatogram is badly tailed, the analytical advantages are quite evident.

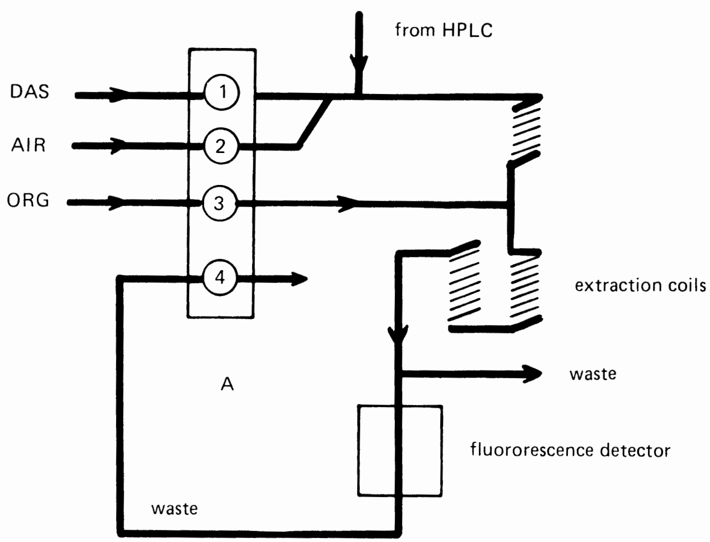

Figure 13 Flow diagram for ion-pair extraction with fluorescence detection. (Reprinted with permission from Ref. 82.)

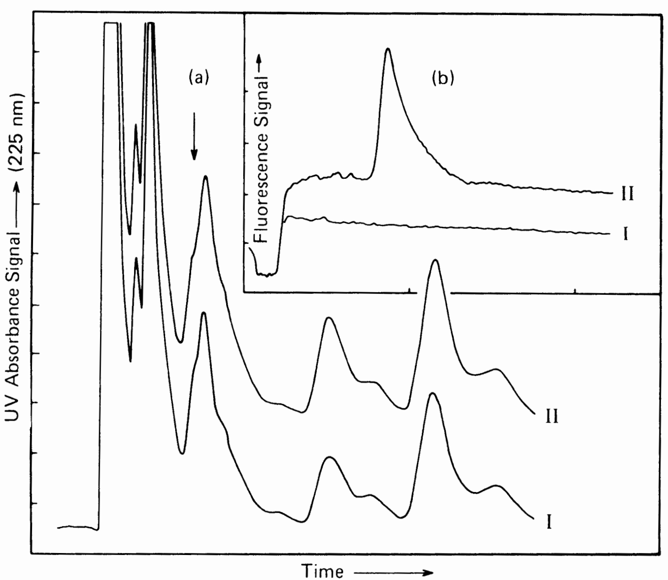

Figure 14 Chromatograms for a urine sample after preconcentration. (a) UV trace at 225 nm; arrow indicates the position of HA, a metabolite of the herbicide atrazine. (b) The ion-pair extraction fluorescence trace. I = blank urine; II = urine spiked with 0.5 ppm HA; sample size, 10 mL; column, RP 18; mobile phase, 25% methanol/0.1 N NaH_2PO_4, pH 3.5; flow rate, 1.0 mL/min.

3. Photolytic Reactors

These reactors represent an ideal in postcolumn chemistry, at least from the simplicity of the design. In a photolytic reactor, the chemical reagent is light; therefore, no fluid reagents need be added to the reactor. The only contribution to band broadening is the length of the reactor, and the variances can be minimized by using deformed tubing [83]. Photolytic reactors have been used for the determination of clomiphene [84], clozebam [85], diethylstilbestrol [86], tamoxifen [87], and the cannabinoids [88]. The reaction for cannabinol (CBN) results in the transformation of CBN [4] to the substituted phenanthrene [5] as shown below in Eq. (7):

$$\mathbf{4} \xrightarrow{h\nu} \mathbf{5} \qquad (7)$$

This chemistry may seem limited only to those compounds for which a suitable fluorophore is the reaction product, but it is not. Recent work by Gandelman and Birks [89] has shown the potential for a very general and sensitive method based on a photolytic reactor. In their method, anthraquinone (AQ) is added to the mobile phase in small quantities. When a reactive solute elutes from the column, a series of reactions (8) involving the triplet state and a free radical mechanism results in the formation of the highly fluorescent dihydroxyanthracence (AQH_2). The solute in the authors' proposed mechanism is isopropanol.

$$\begin{aligned}
&AQ + h\nu \rightarrow AQ^* \text{ (singlet, S)} \\
&AQ^* \text{ (S)} \rightarrow AQ^* \text{ (triplet, T)} \\
&AQ^* \text{ (T)} + (CH_3)_2CHOH \rightarrow AQH\cdot + (CH_3)_2\dot{C}OH \\
&(CH_3)_2\dot{C}OH + AQ\cdot \rightarrow (CH_3)_2C{=}O + AQH\cdot \\
&2AQH\cdot \rightarrow AQ + AQH_2
\end{aligned} \qquad (8)$$

The implications of this chemistry in drug analysis are profound. The authors reported limits of detection in the low nanogram range for aliphatic alcohols, ethers, and aldehydes. It will be only a matter of time before this technique will be applied to various drugs containing these functionalities, that are difficult to determine by other means. The major disadvantage of the method is that the mobile phase must be oxygen free, or the radical intermediates will be scavenged. These

authors, in collaboration with Frei and Brinkman, achieved LODs of 10 ng for digitalis glycosides [89].

4. *Chemiluminescence*

The most important contribution to noise in fluorescence detection is fluctuation of the light source. Since all mobile phases contain some fluorescing impurities, this means the background is constantly and rapidly changing, particularly at high sensitivities. If improper filter combinations are selected, the noise problem can become more severe when scattered light (first or second order) or Raman bands reach the photomultiplier tube. Elimination of the light source could have far-reaching effects in increasing the sensitivity of chromatographic detection.

Chemiluminescence (CL) has become widely known to the general population from American Cyanamid's Cyalume lightsticks. These devices can produce chemically generated light for up to 12 hr. The proposed mechanism of the reaction is as follows (9):

$$\underset{\text{TCPO}}{\text{PhO-}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{-OPh}} + H_2O_2 \longrightarrow \underset{\underline{6}}{\left[\begin{array}{c} \overset{\text{O}}{\overset{\|}{\text{C}}} - \overset{\text{O}}{\overset{\|}{\text{C}}} \\ | \quad\;\; | \\ \text{O} - \text{O} \end{array}\right]} + 2\text{TCP} \qquad (9)$$

$$\underline{6} + \text{Fluorophore} \longrightarrow \text{Fluorophore}^{*} + CO_2$$

$$\text{Fluorophore}^{*} \longrightarrow n\nu + \text{Fluorophore}$$

The 1,2-dioxetanedione [6] is a transient intermediate that has never been isolated.

In 1980, Kobayashi and Imai [65] employed CL for the determination of dansylated amino acids and found LODs of 10 fmol. These same authors reported 25 fmol LODs for fluorescamine-derivatized catecholamines [66]. In 1983, Sigvardson and Birks [67] employed a similar postcolumn reactor for the determination of polycyclic aromatic hydrocarbons.

In our own laboratories, using the CL system depicted in Fig. 15, a limit of detection of 14 fg or 68 amol for aminopyrene was determined. This agrees with work by Birks [67] who noted unusual LODs for amino aromatic compounds. The mobile phase was 75% acetonitrile/25% water and contained 1 g/L tris buffer adjusted to pH 7.5 A μBondapak C_{18} column was used. The peroxide concentration was 1 M in isopropanol, and the TCPO [bis(2,4,6-trichlorophenyl)oxalate] concentration was 1.2 g/L. The column flow-rate was 1 mL/min, and the postcolumn flow-rates were 1.2 and 0.6 mL/min for the peroxide and TCPO, respectively. These conditions are similar to those previously reported by the above-referenced authors. The postcolumn reactor differed in that there was

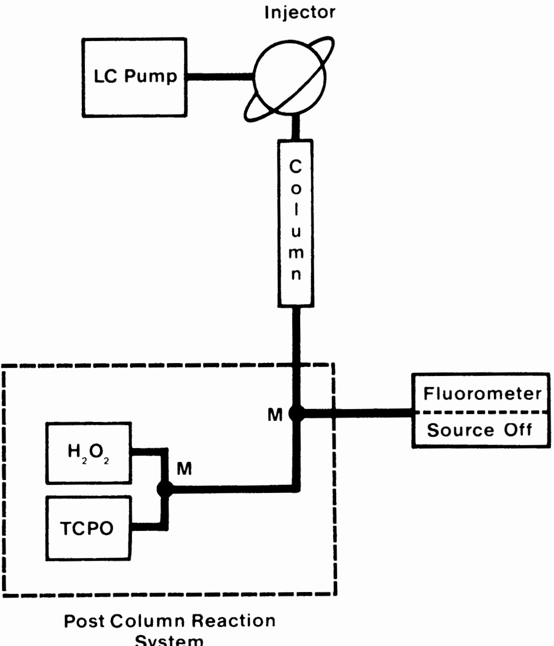

Figure 15 Chemiluminescence system.

no delay coil after mixing the peroxide and TCPO. The purpose of that mixing coil is to minimize noise, but with low-volume vortex tees, mixing noise was not a problem. All of the work previously reported, plus our own, used the Kratos FS 970 detector, which appears well suited for CL.

Thus, CL appears to offer the most sensitive analytical technique known to data that can be performed with conventional and relatively inexpensive equipment. It should give superior results for amino-aromatic compounds, dansyl and fluorescamine derivatives. Hopefully, other native CL compounds will be identified. Generally, compounds that have low energy transitions from ground to singlet state are more easily excited chemically. The major disadvantage of this technique is the relative insolubility of TCPO which may limit the technique to mobile phases, rich in organic modifiers. Other water-soluble CL reagents may resolve that problem.

VII. PHOSPHORESCENCE

Historically, all phosphorescence measurements of analytical significance were required to be performed at cryogenic temperatures. The

long lifetime of the triplet state at room temperature favored radiationless deactivation (quenching) instead of emission. However, over the last few years, several unique techniques for observing room-temperature phosphorescence (RTP) have been developed, and all of these have potential for liquid chromatographic detection.

Although experimentally more difficult than fluorescence, RTP has certain inherent advantages in the total analytical scheme:

1. RTP is selective. This selectivity is a result of two features. First, not all compounds that fluoresce will phosphoresce, and second, phosphorescence emission occurs in a redshifted and less crowded region of the spectrum. The selectivity of RTP has potential for reducing the sample preparation requirements for a particular assay.

2. RTP offers the potential for a native luminescence assay for aromatic compounds that are only weakly fluorescent. This is particularly true for molecules with unshared electron pairs that interact with the π system. For these compounds, intersystem crossing is an important mechanism for deactivation of the singlet excited state.

The oldest of these RTP techniques is solid-substrate phosphorescence [98]. A sample is spotted on a filter paper or similar adsorbant, dried, and the phosphorescence measured. The solid substrate serves to immobilize the solute, thus protecting the molecule from many of the quenching processes that normally occur at room temperature in fluid solution. This technique has applicability to the widest variety of compounds for RTP measurement. Sensitivities are frequently in the subnanogram range. Although seemingly incompatible with liquid chromatographic detection, it is quite possible to design an automated device to perform the necessary processing steps.

The balance of this section will be concerned with three techniques that occur in fluid solution: micelle-stabilized room-temperature phosphorescence (MSRTP), sensitized phosphorescence (SP), and cyclodextrin phosphorescence (CRTP), since they are all directly compatible with LC detection.

A. Micelle-Stabilized Room-Temperature Phosphorescence (MSRTP)

Micelles are aggregates of surfactant molecules that organize in roughly spherical assemblies, once a certain critical micelle concentration (CMC) is obtained. For sodium dodecyl sulfate (SDS), this concentration is 0.008 M in aqueous solution. It has been shown that micellar media can stabilize the triplet state and allow analytically useful RTP to be measured [99-101]. The RTP signal is especially strong of a heavy atom counterion, e.g., thallium, is incorporated into the micellar assembly.

Another feature of micellar solutions is their ability to serve as the mobile phase in liquid chromatography [102,103]. Thus with micellar mobile phases, the MSRTP technique can be used for LC detection [104].

MSRTP is best observed for polycyclic aromatic hydrocarbons (PAH) since these compounds are relatively water-insoluble, spend much of their time in the micellar environment, and are structurally rigid enough to minimize vibrational deactivation of the triplet state. Drugs that are substituted analogues of these PAHs are good prospects for the MSRTP assay. For example, propranolol (Fig. 3) and its 4-hydroxy metabolite, naphazoline, and naproxen are all strong MSRTP emitters. These compounds are substituted naphthalenes, so their MSRTP behavior is predictable.

There are certain disadvantages and limitations of the MSRTP assay that need be overcome before the procedure can be placed in routine use: (a) The mobile phase must be deoxygenated thoroughly. With surfactant solutions, this is somewhat difficult because of excessive foaming. (b) Micellar chromatography is less efficient than conventional reversed-phase separations, though this problem has been largely overcome [105,106].

B. Sensitized Phosphorescence (SP)

Energy transfer in fluid solution is a well-documented and frequent occurrence, particularly when the triplet state is involved because the lifetime is much greater than the rate of energy transfer. Very few molecules exhibit significant phosphorescence in homogeneous (nonmicellar) fluid solution at room temperature. One such exception is biacetyl, [7] a molecule

7

termed "pathologic" by Turro [107]. Biacetyl is probably one of the only nonaromatic molecules that is known to phosphoresce in measurable amounts.

Sensitized phosphorescence is an RTP technique whereby a donor (analyte) molecule is excited and transfers its triplet energy to an acceptor (biacetyl) whose phosphorescence emission is measured. There are four important criteria that must be fulfulled for a successful SP procedure.

1. The triplet energy of the donor must be greater than the triplet energy of the acceptor. This is found for many molecules since the emission of biacetyl peaks at about 515 nm.

2. The lifetime of the donor triplet state must be greater than the rate of energy transfer. This is often a limiting step.
3. The donor triplet state must be sufficiently populated. This, too, is often limiting.
4. The donor must absorb light at a wavelength away from the acceptor. This is seldom limiting.

Thus SP offers a sensitive and selective technique for the determination of weakly fluorescing compounds (because of intersystem crossing), without micelles, that can be used for LC detection. The pioneering work in this field, from which the above material was extracted, was performed by Donkerbroeck and co-workers [108-110].

Some of the problems that are encountered in the MSRTP technique are solved by SP. Since surfactants are not employed, deoxygenation is less of a problem and the chromatographic efficienty is maintained. Conventional LC mobile phases, generally acetonitrile/water mixtures, are ideal media for the energy transfer. Water-soluble molecules, which will not partition into a micelle, can still be detected by SP. However, compounds like propranolol are better measured by MSRTP since their SP response is negligible, presumably due to the short lifetime of the triplet state.

Although most drugs will not give SP, compounds like cocaine, phenobarbital, estradiol, terbutaline (Brethine), and dihydrocodeine (Didrate) are all strong sensitizers [111]. It was recently reported that phenothiazines will quench biacetyl emission, and this is analytically useful [112]. Previous studies with polychlorinated biphenyls have shown picogram LODs, and similar sensitivities may be found for the aforementioned and other drugs.

C. Cyclodextrin RTP

The newest and perhaps most useful RTP technique uses cyclodextrins to stabilize the triplet state. Cyclodextrins are macrocyclic polymers of glucose, composed of α-(1,4)-glycosidic linkages of D(+)- glucopyranose units. These molecules are the end products of the bacterial degradation of starchs. The most abundant and studied molecules are the α-, β-, and γ-cyclodextrins, formed by six, seven, and eight glucose units, respectively [113]. Such a cyclic arrangement of glucose monomers forms a torus or "doughnut" configuration with a molecular cavity of a specific internal diameter. The interior of the torus is hydrophobic but contains one or more molecules of water in aqueous solution. This water is thermodynamically high in energy and can easily be displaced by a more hydrophobic species. This phenomenon gives cyclodextrins their unique property of forming inclusion complexes with a variety of organic and inorganic molecules [114]. The driving force for this complexation has been attributed to Van der Waals forces, hydrogen bonding, and the release of cyclodextrin strain

energy [115]. The interactions between the cyclodextrin and guest molecule are so strong that even if the guest is too large to be fully accommodated in the cyclodextrin interior, complexation may still occur.

Like micelles, cyclodextrins have also been used as mobile phases in liquid chromatography [116]. The applications of cyclodextrins in industrial processing and analytical chemistry have been reviewed by Hinze [117].

The nature of the inclusion complex has a favorable impact on the luminescence properties of guest solutes. The reduction of quenching mechanisms enhances fluorescence [118] and allows the observation of analytically significant RTP [119].

Scypinski and Cline Love [120] have studied cyclodextrin RTP of polycyclic aromatic hydrocarbons in an aqueous environment containing small amounts of dibromoethane The dibromoethane serves as a heavy atom solvent to enhance spin-orbit coupling. This promotes intersystem crossing, which increases RTP. These same authors found tht the RTP is less oxygen-sensitive compared to MSRTP or SP, though optimum sensitivity is found in an oxygen-free environment. This implies that the molecule is tightly held in the cyclodextrin matrix. Further evidence for this feature is the observation of enhanced spectral fine structure compared to MSRTP.

Though cyclodextrin RTP has not yet been applied to a drug analysis, there are obvious applications in this area. Limits of detection for aromatic molecules are in the subpicogram range, making this the most sensitive of the RTP methods. In combination with chromatography, this technique may provide the analytical simplicity and sensitivity that most of the other RTP measurements lack.

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8

LIQUID CHROMATOGRAPHY-ELECTROCHEMISTRY: POTENTIAL UTILITY FOR THERAPEUTIC DRUG MONITORING

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I. INTRODUCTION TO LIQUID CHROMATOGRAPHY-ELECTROCHEMISTRY (LC-EC)

Chromatography and electrochemistry are the two most widely used analytical tools which (a) are heterogeneous, and (b) require an understanding of the mass transport of molecules. Experimentally, the combination provides a powerful tool for trace determinations, and is the fastest growing electroanalytical technique. Figure 1 illustrates the components of a typical LC-EC system. Both chromatography and electrochemistry have long been used in clinical laboratories for specialized applications. With few exceptions (determination of blood oxygen by amperometry), both methodologies have continued to present too many reliability problems for use in smaller laboratories by less skilled personnel.

Liquid chromatography is complex in some of its details, but the technique is very easy to understand from an operational point of view. The goal is to move different compounds or ions through a system at different rates of speed so that they exit from the system at different times. The objective of the chromatography for determining substances in complex biofluids is really to overcome the inability of the detector to distinguish individual chemical substances. If ideal detectors were available, there would be no reason to employ chromatography for analytical purposes. The chance of this happening for samples in the liquid phase is extremely unlikely due to the broad distribution of energy states resulting from collisions with the

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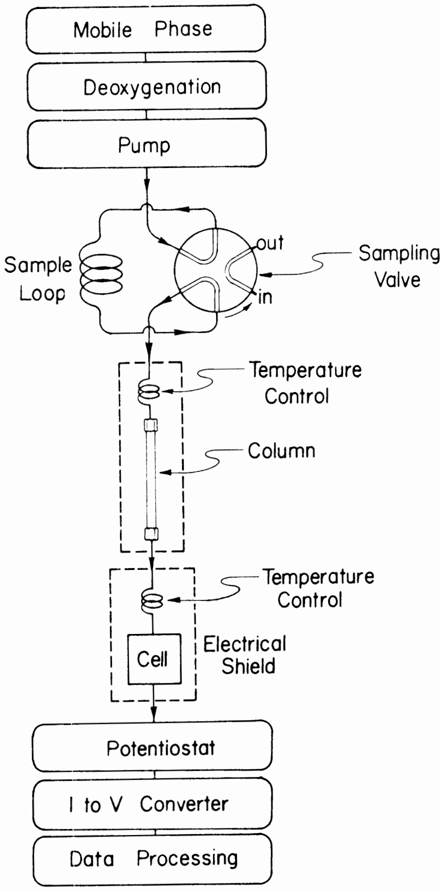

Figure 1 Components of a liquid chromatography-electrochemistry system. (Reproduced with permission of Bioanalytical Systems, Inc.)

solvent. Optical spectra and voltammetric curves tend to be "spread out" to such a degree that the response for an individual compound is likely to overlap with that for many others. This may not be a problem when light absorbance or amperometry is used to study very simple solutions; however, in biomedical samples a separation step is almost always needed. One may think of the chromatograph as a sample preparation tool for the detection scheme used. This philosophy applies perfectly well to the familiar blood oxygen electrodes, where a hydrophobic membrane "separates" molecular oxygen from a very complex sample.

A chromatographic detector should have an active volume which is small relative to the volume occupied by the concentration zone passing from the column [1]. It should also be able to respond rapidly in order to accurately represent the shape of the zone. At this writing, liquid chromatographic peaks with volumes well below 100 μL are becoming quite common. Often the peak width is measured in seconds! Obviously not all liquid chromatographs need be used with such highly efficient columns, and many useful assays still result in much wider peaks.

The most popular liquid chromatography detectors are those which operate on physical principles (e.g., absorbance or emission of light or measurement of refractive index changes). Unlike these instruments, the electrochemical detector (ECD) involves a chemical transformation at a surface. This reaction involves a transfer of electrons and can therefore be followed by measuring current as a function of time (Fig. 2). The current is an instantaneous measure of the *rate* of an electrochemical process (dependent on how many electroactive molecules or ions strike the electrode surface per unit time and the proportion of these which release or gain electrons in the process). The most popular electrochemical detector cells are thin-layer devices in which the active electrode(s) is imbedded in the wall of a channel formed by two blocks pressed around a thin gasket (Fig. 3). The active volume of

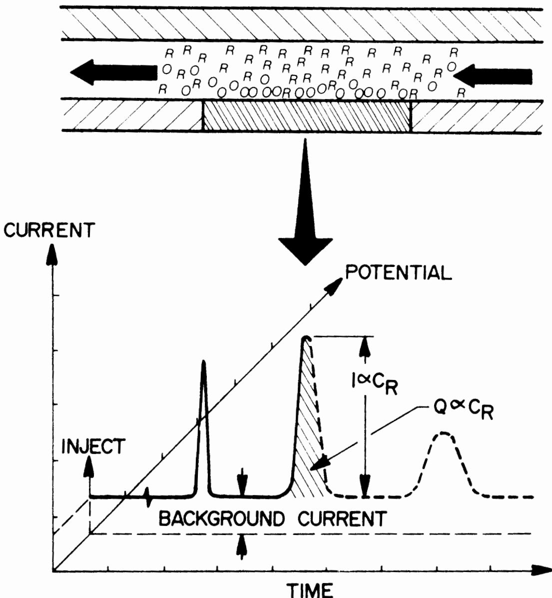

Figure 2 Principles of electrochemical detection. (Reproduced with permission of Bioanalytical Systems, Inc.)

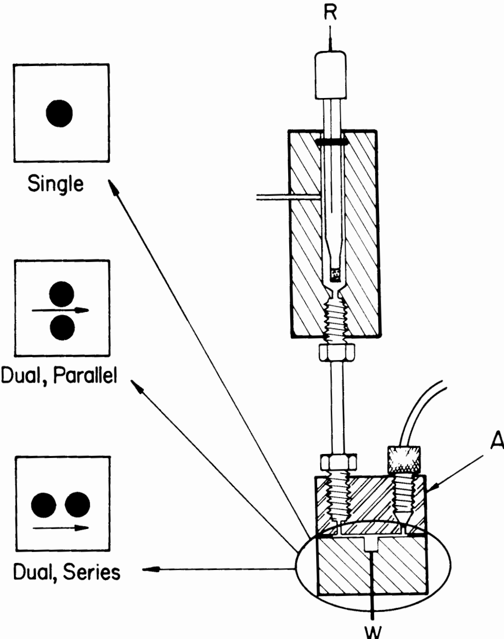

Figure 3 Thin-layer electrochemical flow cell. (Reproduced with permission of Bioanalytical Systems, Inc.)

such cells is typically between 0.1 and 1 μL, and therefore the shape of the chromatographic concentration profile is not disturbed even for the most efficient commercially available columns (particle diameter averaging 3 μm).

Electrochemical detection is based on controlled potential amperometry. A predetermined potential difference (usually between +1.3 and −1.2 V and dependent on the redox behavior of the substance to be detected) is applied between the reference and working electrodes. Simply stated, the applied potential serves as the driving force for the electrochemical reaction. As the potential of the working electrode relative to the reference becomes more positive, the surface becomes a better oxidant (electron sink). Conversely, when the applied potential is more negative, the electrode acts as a stronger reductant (electron source). This is illustrated graphically in Fig. 4, which illustrates the axes used to plot voltammetric information (current vs. voltage curves).

Suppose that an oxidizable drug passes into the flow cell from the column, and the applied potential is set at a sufficiently positive value for its oxidation. As the solute band passes over the electrode sur-

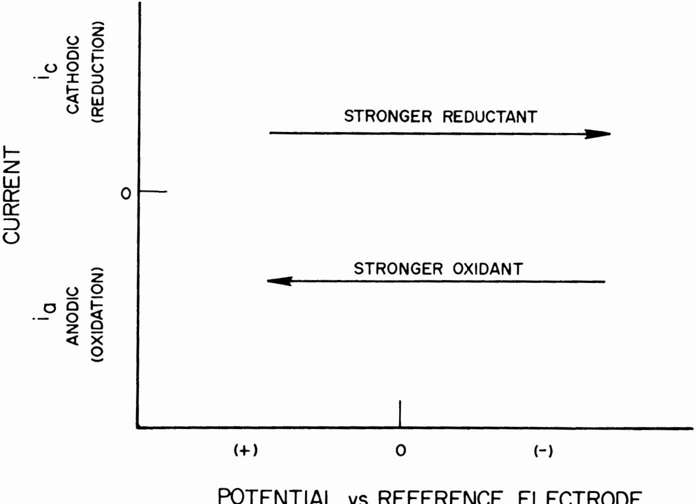

Figure 4 The voltammetric axes. Electrochemical data plotted on these axes reveal thermodynamic and kinetic information useful for optimizing LC-EC. (Reproduced with permission of Bioanalytical Systems, Inc.)

face, those molecules immediately adjacent to the electrode surface will be oxidized in a heterogeneous transfer of electrons. The current that results from this exchange of electrons with the surface is monitored as a function of time. Since the rate of material conversion by the electrochemical reaction (moles per second) is proportional to the instantaneous concentration, the current will be directly related to the amount of compound eluted as a function of time. If chromatographic conditions (mobile phase, flow-rate, temperature, etc.) are carefully controlled, then amperometric detection is quite precise. Quantitative data can be obtained at the picomole level (total injected amount) for many compounds. Since only those molecules adjacent to the electrode surface are reacted, it is not surprising that the conversion efficiency (% of reactant which actually reacts before passing over the electrode) of the thin-layer amperometric transducer is typically only 3-5% under normal operating conditions (a coulometric detector would convert 100%).

Frequently, the amount reacted is on the order of 10^{-15} mol! For example, in the case of a molecule with a molecular weight of 200 undergoing a two-electron transfer, only 5×10^{-13} g of sample is converted into product for quantitation at a signal-to-noise (S/N) ratio of 5. Initially, it would seem worthwhile to increase electrode surface area and thereby increase the conversion efficiency. Unfortunately, the conversion efficiencies of both the analyte as well as the background

electrolyte are increased, and the concomitant improvement in (S/N) is not realized. In fact, S/N decreases with increased area for reasons outside the scope of this introduction.

The choice of the electrode surface is critical to successful LC-EC operation. Obviously the surface should be physically and chemically inert to the mobile phase at the chosen applied potential. Three electrode surfaces have found greatest utility: glassy carbon, carbon paste, and mercury. The most versatile choice is glassy carbon. It has excellent resistance to nearly any solvent used in liquid chromatography and may be used over a wide potential range. Mercury provides an extended negative range of potential but is very limited in the positive direction. Conventional, dropping mercury electrodes are not amenable to the low dead-volume, thin-layer design. A better alternative is to employ a mercury amalgam on a polished gold substrate. The mercury film can be made quite thin and very smooth. Mercury is better than glassy carbon when dealing with substances difficult to reduce. It is also the electrode of choice for many sulfur-containing compounds.

II. ELECTROACTIVE DRUGS

A. Overview

Molecular structure is, of course, the primary determinant for electroactivity, just as it is for spectroscopy. The accessibility of various filled and unfilled molecular orbitals ultimately determines the thermodynamics and kinetics of the electrode process. As for spectroscopy, there is a great body of empirical information which can be used as a basis for predicting the behavior of individual compounds. In examining a candidate drug or metabolite, the chemist will want to ask several key questions. What functional groups are present? Does the parent structure permit delocalization of the added positive or negative charge? Are there substituents present in the molecule that enhance or detract from electroactivity? Is the redox reaction pH dependent? What is the solubility? All of these factors are important considerations in assessing electroactivity for LC-EC or any other electrochemical technique. Among the many electroactive organic compounds, the following classes of substances are frequently ideal candidates for LC-EC: phenols (especially hydroquinones and catechols), aromatic amines, thiols, nitro compounds, and quinones. There are many other classes which fit, as well as some unique compounds such as ascorbic acid, uric acid, phenothiazines, and NADH. Derivatization is now being used to make some compounds better candidates for LC-EC. For example, nitrophenyl groups have been added to amino acids, carbonyl compounds, carbohydrates, and carboxylic acids.

Cyclic voltammetry rapidly provides useful information preliminary to LC-EC [2]. This very convenient stationary-solution experiment

easily duplicates those conditions (electrode material, electrolyte, etc.) found in the LC-EC detector cell. Analogous to generating a UV spectrum, cyclic voltammetry discerns oxidation or reduction behavior as a function of applied potential. Figure 5 illustrates a low-cost cyclic voltammograph, and Fig. 6 depicts a typical tracing for the easily oxidized drug α-methyldopa. The "cyclic voltammogram" is truly the electrochemical equivalent of an optical spectrum and is used in much the same way.

B. Selectivity in LC-EC

The liquid chromatographic process separates compounds according to the different rates at which they migrate through the column. While modern LC columns can achieve remarkable feats of selectivity in a very short time, they are rarely adequate by themselves to achieve a completely satisfactory result. Often a sample cleanup step is required prior to injecting material on the column. Most of the procedures outlined in this book require a cleanup step. This would typically consist of one or more liquid-liquid or liquid-solid extractions carried out in a batch mode.

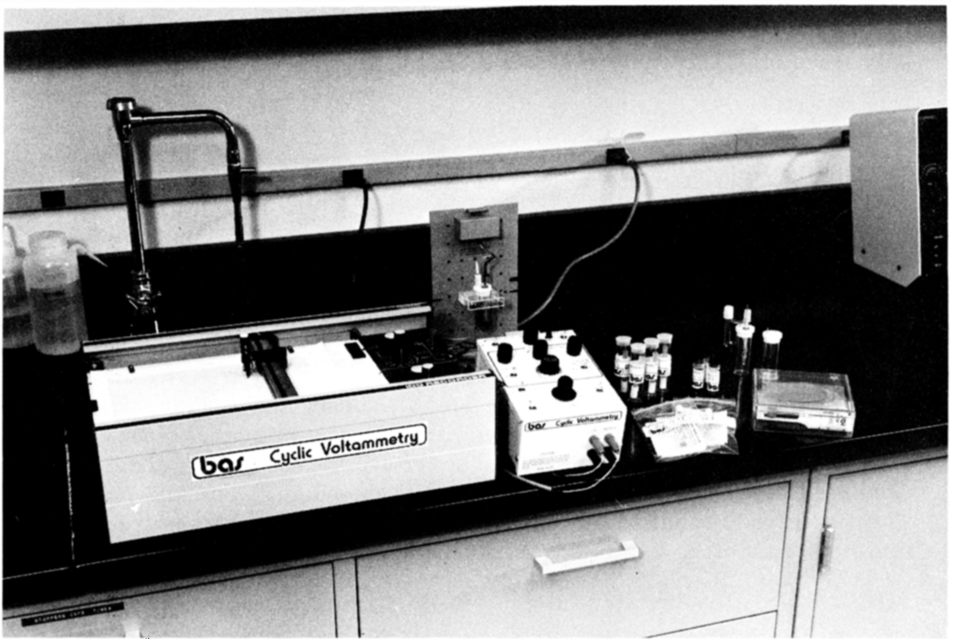

Figure 5 Cyclic voltammograph. (Reproduced with permission of Bioanalytical Systems, Inc.)

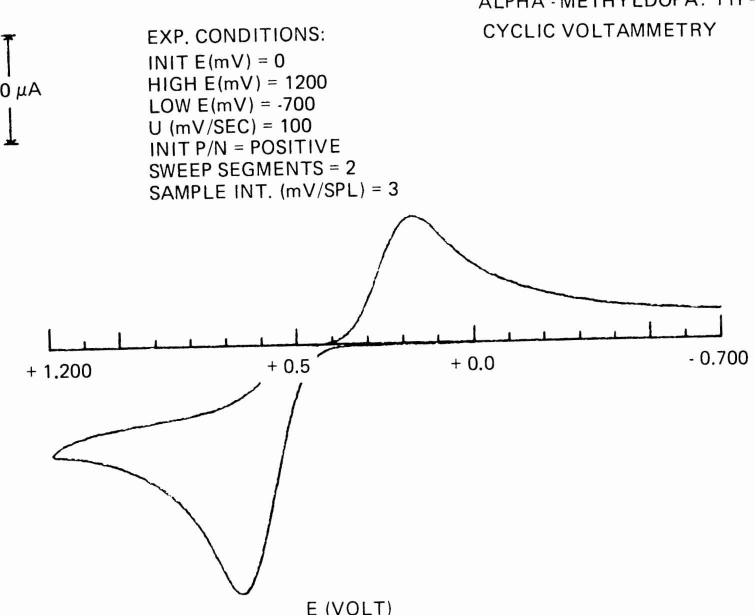

Figure 6 Cyclic voltammogram for α-methyldopa. (Reproduced with permission of Bioanalytical Systems, Inc.)

The selectivity of the column is also commonly augmented by the detector. This is a real strength of LC-EC. While a "universal detector" could be useful for some simple problems, a "selective detector" is more likely what is needed. The LC-EC detector is a tunable device which permits the selectivity to be adjusted by changing the applied potential. The choice of electrode material and mobile-phase composition also can influence the selectivity. To fully understand how this works, it is important to be familiar with "hydrodynamic voltammetry," a technique which is also the basis for familiar amperometric and coulometric titrations. Hydrodynamic voltammograms are plotted on the same axes as are cyclic voltammograms. The difference results from the fact that the solution is moving, and the products of the electrode reaction are rapidly swept away from the electrode and therefore cannot be examined by reversing the potential scan.

C. Representative Selection of Electroactive Drugs

1. *Oxidative Applications [3]*

Phenolic substances: These are for the most part readily oxidized at a graphite electrode. The oxidation potentials for phenols vary widely with structure, and some (hydroquinones and catechols) are far more readily oxidized than others. Many compounds of pharmacological interest (catecholamines, pharmaceuticals) and industrial interest (antioxidants, antimicrobials, agricultural chemicals) are phenolic, and trace determinations based on LC-EC are now quite popular. Aldomet (α-methyldopa) is a hydroquinone (a catechol) representative of the more easily oxidized phenols. It undergoes a clean two-electron oxidation to an *o*-quinone:

HO, HO, NH_3^+, COOH, CH_3 ⟶ O, O, NH_3^+, COOH + 2e + $2H^+$, CH_3

Aromatic amines: Like phenols, aromatic amines are oxidized at a graphite electrode over a wide range of oxidation potentials. Some compounds (phenylenediamines, benzidines, and aminophenols) are ideal candidates due to their very low oxidation potentials, and numerous applications have been developed. The analgesic acetaminophen is an ideal example of an aminophenol which readily oxidizes to an acetylated quinone imine:

$HN\overset{O}{\overset{\|}{C}}CH_3$, OH ⟶ $N\overset{O}{\overset{\|}{C}}CH_3$, O + 2e + $2H^+$

Thiols ("sulfhydryls" or "mercaptans"): These compounds are very easily oxidized to disulfides in solution, but this thermodynamically very favorable redox reaction occurs only very slowly at most electrode surfaces (e.g., glassy carbon). Therefore, LC-EC methods for thiols usually depend on the unique behavior of these compounds at a mercury electrode surface at about +0.10 V (a very low potential). The reaction involves formation of a stable complex between the thiol and mercury. In a formal sense, the mercury and not the thiol is oxidized:

$$2\ RSH + Hg \longrightarrow Hg(RS)_2 + 2e + 2H^+$$

This approach has been used to determine the amino acid cysteine, the tripeptide glutathione, and the pharmaceuticals penicillamine and captopril. Besides thiols, many other sulfur-containing compounds are good candidates for LC-EC.

Miscellaneous oxidizable compounds: A number of unique substances have been studied by oxidative LC-EC. Being an excellent reducing agent, ascorbic acid is easily detected with excellent selectivity in very complex biological samples:

$$\text{Ascorbic acid} \rightleftharpoons \text{Dehydroascorbic acid} + 2H^+ + 2e$$

Similarly, uric acid is readily detected in biological materials. The important enzyme cofactor, NADH, is readily oxidized at carbon electrodes and provides a unique opportunity for enzyme immunoassays coupled to LC-EC:

$$\text{NADH} \longrightarrow \text{NAD}^+ + 2e + H^+$$

Some heterocycles of pharmaceutical interest (phenothiazines, imipramine) are also uniquely applicable. Phenothiazines undergo a very clean, one-electron oxidation to a cation radical:

$$\text{Phenothiazine} \longrightarrow \text{Phenothiazine}^{+\bullet} + e$$

2. *Reductive Applications [3]*

Quinones: These are among the best behaved organic compounds to undergo redox reactions in aqueous solutions. There are a reasonably large number of synthetic and natural products containing the quinone moiety, and many of these are excellent candidates for selective determination by LC-EC. Unfortunately, some of the most important of these compounds (vitamin K_1) are extremely hydrophobic, due to the presence of long hydrocarbon side chains and are therefore quite difficult to study by reversed-phase LC. A number of antibiotics used in chemotherapy are quinones. For example, pharmacokinetic studies

of doxorubicin can benefit from the following electrochemical reaction used in an LC-EC method:

$$\text{Doxorubicin} + 2e + 2H^+ \longrightarrow \text{hydroquinone form}$$

Nitro (and nitroso) compounds: These have been among the most extensively investigated by both organic and analytical electrochemists. Aromatic nitro and nitroso compounds are very readily reduced at both carbon and mercury electrodes, but other compounds such as nitrate esters, nitramines, nitrosamines, and nitrosoureas are often good candidates as well. Often the selectivity is extremely good in biological and environmental samples because the nitro group is rare in nature, and few other organic compounds are so easily reduced. A good example is the popular antibiotic chloramphenicol, which can be easily determined in blood using a four-electron reduction to the corresponding hydroxylamine:

$$\text{Ar-}NO_2 + 4e + 4H^+ \longrightarrow \text{Ar-}HNOH + H_2O$$

(Ar = C_6H_4–HCOH–HCNHC(=O)$CHCl_2$–H_2COH)

Reagents containing the aromatic nitro group have frequency been used to derivatize amines, aldehydes, ketones, and carboxylic acids, etc., to improve their characteristics for determination by absorption spectroscopy. The same or closely related reagents are now being used to provide an electrochemically reactive handle for many of these compounds. Although it is true that aldehydes and ketones can be electrochemically reduced and alkyl amines and carboxylic acids can be electrochemically oxidized, the energy required to carry out these well-known reactions is at present far too great to permit development of a successful LC-EC trace determination procedure without derivatization. This subject will be covered in some detail in Sec. IV.B.

Miscellaneous reducible compounds: A number of organometallic compounds show promise for LC-EC study, and a few have already been examined in detail. More highly conjugated organic compounds, such as α,β-unsaturated ketones and imines, are occasionally good candidates, but at this time ultraviolet (UV) detectors frequently outperform electrochemical detectors for such systems. To data, there have been only a few reported LC-EC studies of metal ions or metal complexes. The disulfides derived from in vivo oxidation of thiol-based drugs can be detected by electrochemical reduction of the disulfide to the thiol and detection of the latter by oxidation as described. This approach uses the dual-series arrangement with two mercury film electrodes.

III. REVIEW OF APPLICATIONS

A. Analgesics

As a group, analgesics have been the drugs most widely studied by LC-EC techniques (see Fig. 7). They are typically oxidizable aromatic compounds which are either phenols and/or amines. It is not surprising to find that most attention has been paid to acetaminophen, salicylates, and morphine (see Table 1). The interest in using LC-EC to determine these substances has been primarily for research purposes, rather than for routine TDM, where more familiar methods often suffice. The metabolism of aromatic xenobiotics is frequently ideal from an electrochemical perspective because the most interesting pathways (from a toxicological viewpoint) often lead to products which are more easily oxidized than the initial reactant. This means that minor drug metabolites can often be detected by LC-EC at lower concentrations than the drug itself; exactly what is needed. Determination of secondary metabolites often provides useful hints as to the mechanism by which toxic reactive intermediates are formed. For these reasons, LC-EC is used in metabolic research involving biological fluids, organ perfusates, tissue samples, and subcellular fractions (e.g., liver microsomes). Figure 7 illustrates the determination of a variety of analgesics in human blood serum.

B. Antibiotics

LC-EC assays of antibiotics often have the advantage over other previously reported methods due to improved specificity combined with lower limits of detection. Traditionally, these compounds have been analyzed by some form of bioassay which has no specificity, and interferences from both active metabolites and other antibiotics are likely. A number of LC methods employ UV detection, but these can be troublesome due to poor detection limits and extensive sample

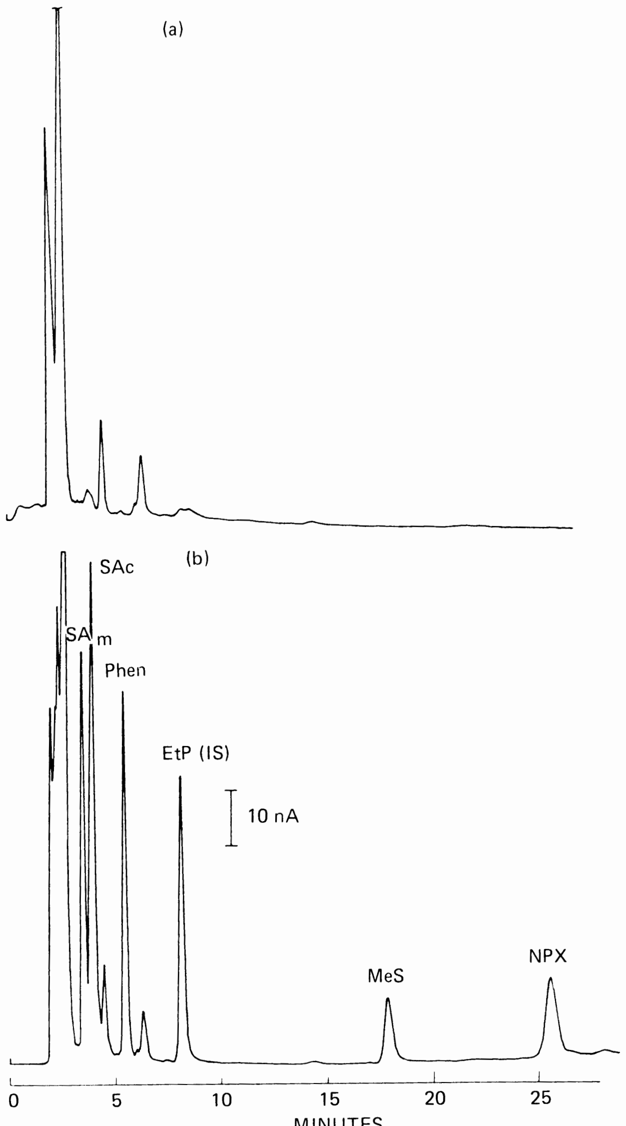

Figure 7 Isocratic LC separation of several analgesics in a spiked serum sample: (a) serum blank; (b) serum sample. Mobile phase = 0.05 M ammonium monochloroacetate (pH 3.2), 0.1 M $NaClO_4$ in MeOH:H_2O (50:50), Biophase ODS column. Detector potential = +1.15 V. Approximately 40 ng of each injected: salicylamide (SAm), salicyclic acid (SAc), phenacetin (Phen), ethylparaben (EtP), methylsalicylate (MeS), naproxen (NPX). EtP is used as the internal standard. (Reproduced with permission of Bioanalytical Systems, Inc.)

Table 1 Analgesics[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Acetaminophen	B	RP	CP	+0.65	20 ng/mL	12
Acetaminophen	B	FIA	CP	+0.70 (SCE)	10 mg/mL	6
Acetaminophen	DF,B,U	RP	CP	+1.00	1 mg/mL (U) 0.1 mg/mL (S)	9
Acetaminophen	B	RP	CP	+0.70	0.2 mg/mL	10
Acetaminophen	B	RP	GC	+0.80	0.005 mg/mL	11
N-acetyl-*p*-quinoneimine (metabolite of acetaminophen)	T	RP	CP/GC	+0.75	~ 10^{-5} M	4
Acetaminophen metabolites	U	RP	CP	+0.60	−1.1-0.5 ng	8
Acetaminophen metabolites	U	RP	CP	+1.20	0.15-0.40 ng	5,7
Acetaminophen	U,B	RP	GC	+0.7, +1.1	NK	14,21
Salicyclamide						
Phenacetin						
Methyl salicylates						

Codeine						
Naproxen						
Naloxone	B	RP	GC	+0.75	1 ng	15
Nalbuphine						
Morphine	B	RP	GC	+1.00	1 ng/mL	16
Morphine	T	RP	GC	+0.725	1 ng	17
Morphine	B	RP	GC	+0.70	0.5 ng	18
Morphine	CSF,B	RP	GC	0.79	1 ng/mL	19
Naloxone	T	RP	GC	+0.60-0.80	NK	20
Meptazinol	B	FIA	GC	+1.20	100 pg	13
Morphine	B	RP	GC	+0.65	1 ng/mL	22

[a]Abbreviations used: Sample: B = blood, DF = dosage form, T = tissue, U = urine, CSF = cerebrospinal fluid, M = human breast milk, S = standard. LC mode: NP = normal phase, RP = reversed phase, IE = ion-exchange, FIA = flow injection analysis (no column used). W = working electrode material: CP = carbon paste, GC = glassy carbon, Hg = mercury, Au = gold, PG = porous graphite. E = applied potential used (not necessarily related to the thermodynamic oxidation potential): MDA/MDC = minimum detectable amount or concentration if reported (a very rough guide due to numerous factors which can influence this figure, which in many cases, is reported in subjective terms); NK = not known.

cleanup procedures. Unfortunately, many antibiotics are *not* electrochemically reactive, and LC-EC may not be a viable option.

Chloramphenicol is an antibiotic that can be assayed conveniently by LC-EC at negative potentials at a mercury film electrode, like other compounds containing an aromatic nitro group. Since there are very few reducible compounds found naturally in blood, this reductive LC-EC assay should be highly selective for such exogeneous compounds (Fig. 8). Furthermore, this is an ideal compound to run using dual-

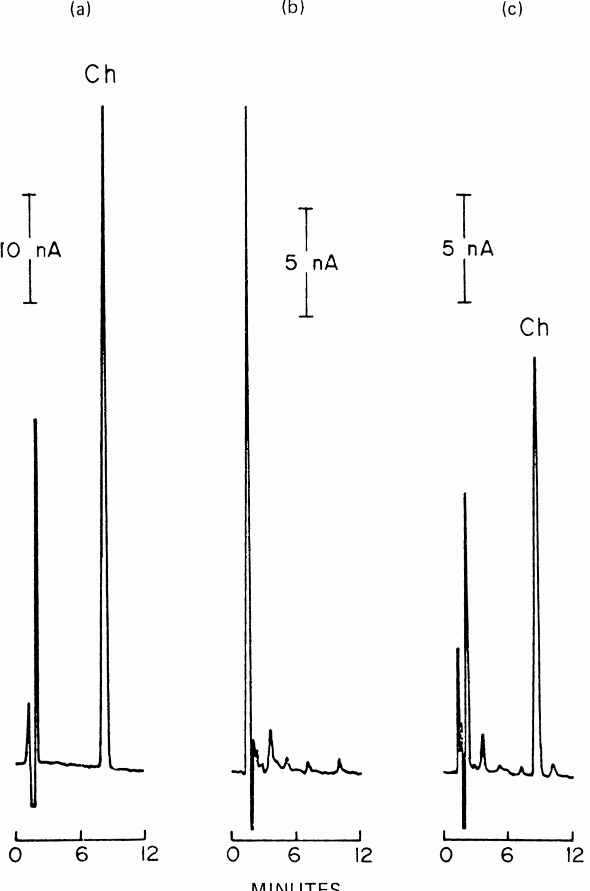

Figure 8 Chloramphenicol in plasma. (a) Chromatogram of chloramphenicol standard, 295 pmol. (b) Unspiked drug-free plasma blank from healthy subject. (c) Same plasma spiked with 0.7 μg/mL chloramphenicol. (Reproduced with permission of Bioanalytical Systems, Inc.)

series electrochemical detection, with the upstream electrode set at -0.85 V (reducing $-NO_2$ group to $-NHOH$) and the dowstream electrode set at +0.50 V (oxidizing $-NHOH$ to $-NO$). Utilizing the new current shunt feature on the dual-electrode detectors, one could easily perform this assay while avoiding mobile-phase deoxygenation. Both trimethoprim and sulfonamides can be detected utilizing LC-EC methodology at a greater sensitivity than is possible with UV detection, although the applied potential required is high, generally about +1.1 V to +1.2 V.

Tetracyclines have several aromatic hydroxyl moieties which make them amenable to electrochemical detection. Although LC-UV had been tried with these compounds, plasma and urine extracts produced high backgrounds which limited the sensitivity of the procedure. LC-EC was found to be ideally suited to the determination of nanogram amounts of β-cetotetrine in biological fluids requiring a low applied potential of +0.58 V [25].

Amoxicillin in dosage forms has been assayed by LC-EC, and could theoretically be assayed in body fluids if a good sample extraction process could be developed. Amoxicillin has a high water solubility which precludes its extraction into organic solvents. A trace enrichment technique may prove to be useful here.

Erythromycin, a macrolide antibiotic, has been shown to be electrochemically active as well, although details of its electrochemical mechanism have yet to be elucidated.

Enviroxime is a highly specific inhibitor of the multiplication of rhinovirus in tissue cultures and is currently under evaluation as a treatment for the common cold. A sensitive and selective determination of this compound has been reported utilizing LC-EC with an applied potential of -0.85 V for detection [28].

While electrochemical detection of antibiotics is relatively early in its development, there are a number of promising applications (see Table 2 and the following section on chemotherapeutic agents).

C. Anticancer Drugs

Many of the diverse chemical agents used to treat various cancers have favorable redox properties for very selective LC-EC methods (see Table 3). Due to the experimental nature of many of the compounds, their toxicity, and their use in critical-care situations, it is especially important to monitor the bioavailability of the drugs in individual patients. Most of this work is focussed on specialized insititutions where new chemotherapy protocols are explored. The efforts of Sinkule, Evans, and co-workers at St. Jude Children's Research Hospital are expecially noteworthy in this regard [30,32]. Figure 9 is representative, but newer columns provide better resolution and detection limits in less time. Determination of platinum complexes is more difficult, but reductive LC-EC can provide good results (Fig. 10).

Table 2 Antibiotics[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Chloramphenicol	B	RP	Au/Hg	−0.85	NK	23
Trimethoprim	B,U	RP	GC	+1.20	2 pmol	24
Sulfonamides	T	RP	NK	+1.10	10 ng/g	25
β-Cetotetrine	B,U	RP	CP	+0.58 (SCE)	0.05-0.10 ng	26
Amoxicillin	DF	RP	CP	+0.94 (SCE)	42 ng	27
Enviroxime	P,U,T	RP	GC	+0.85	5 ng/mL	28
Erythromycin	B	NK	PG	+0.80	50 ng/mL	29

[a]Abbreviations used: see Table 1.

Table 3 Anticancer Drugs[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Doxorubicin	B	RP	GC	+0.65/+0.75	10 ng/mL	30,32
Daunorubicin						
Teniposide (VM26)						
Etoposide (VP-16)						
Bisantrene						
Etoposide	B	RP	GC	+0.50	250 pg	41
Daunorubicin	B	RP	GC	+0.65	8 ng/mL	31
Mitomycin C	B,U	RP	Hg	−0.60	250 pg	33
Aclacinomycin A	S	NP	GC	−0.50	NK	34
Carminomycin						
Marcellomycin						
Daunorubicin						
Adriamycin						
Methotrexate	B	RP	NK	+0.80	2×10^{-8} M	35
Procarbazine HCl	B,U	NP	CP	+0.75	2 ng	36
cis-Platin	B	RP	Hg	−0.10	0.2 ng	37,39,40
5-Methyltetrahydrofolic acid	B,CSF	RP	GC	+0.30	0.9 ng/mL	38

[a]Abbreviations used: see Table 1.

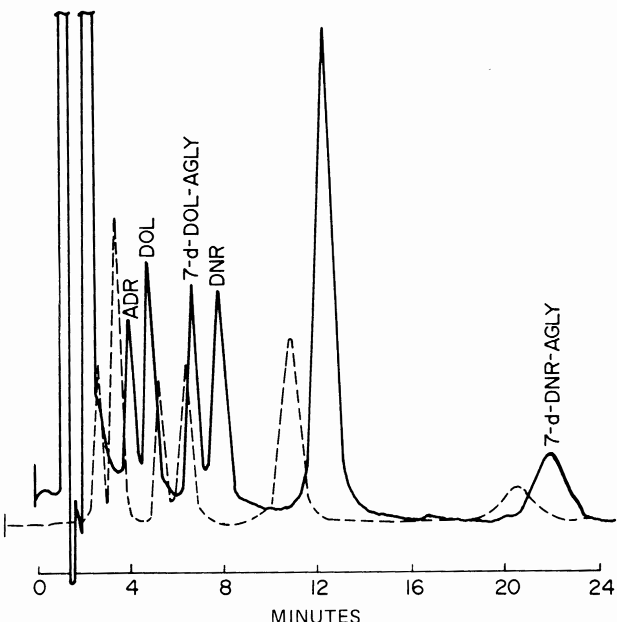

Figure 9 Anthracyclines in plasma. Separation of a mixture of daunorubicin and metabolites in plasma by HPLC, with quantitation by fluorescence (---) and electrochemical (——) methods. The column was a μBondapak C_{18} (30 cm × 3.9 min), and the solvent contained acetonitrile:water (28:72 by volume) adjusted to pH 4.0. Mobile phase flowrate was 2.0 mL/min. The sample was a mixture of ADR (3.8 min), DOL (4.8 min), 7-deoxy-DOL-AGLY (6.5 min), DNR (8.0 min), DNR-AGLY (12.6 min), and 7-deoxy-DNR-AGLY (22.3 min). (Reproduced with permission of BAS Press.)

D. Beta Mimetics and Beta Blockers

Beta mimetics and beta blockers have proved to be efficacious for a wide range of therapeutic purposes due to their various receptor binding affinities, and thus are in widespread use. The tocolytic agents ritodrine, terbutaline, and isoxsuprine are used as myometrial relaxants to help prevent premature labor; isoxsuprine and nylidrin as vasodilators; terbutaline, metaproterenol, and salbutamol as bronchodilators. Previously, assay of these drugs had been limited to radioimmunoassay, which had adequate sensitivity but was prone to variability in the data (attributed to cross-reactivity), and run-to-run variability, as well as a lengthy turnover time.

Liquid chromatography with electrochemical detection for these compounds provides added advantages in that several compounds can

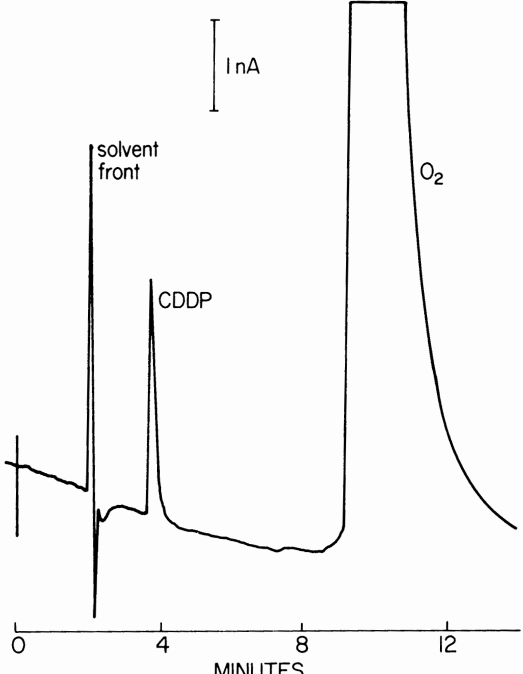

Figure 10 *cis*-Platin (CDDP) standard. A typical reductive LC-EC chromatogram at the 0.63 ppm concentration level, using a reversed-phase C_{18} analytical column, with a mobile phase of 0.01 M acetate buffer (pH = 4.6) plus 0.15 mM HTAB. The Au/Hg working electrode is operated at an applied potential of −0.1 V. Under these LC-EC conditions, there is an observed retention time of 3.6 min, with oxygen well-resolved and appearing at about 9.33 min. The minimum detection limit for *cis*-Pt, using a signal:noise ratio of 3:1, has been determined to be about 10 ppb. (Reproduced with permission of BAS Press.)

be rapidly determined in a single analytical run with adequate sensitivity (Fig. 11). Most of these assays can be performed quite conveniently with either reversed-phase or ion-exchange chromatography. Applied potentials required for detection are slightly higher than for catecholamines (see Table 4).

E. L-Dopa and Related Compounds

The biogenic amines and their analogues are ideally suited to LC-EC. There are many hundreds of publications in this area [3]. The anti-

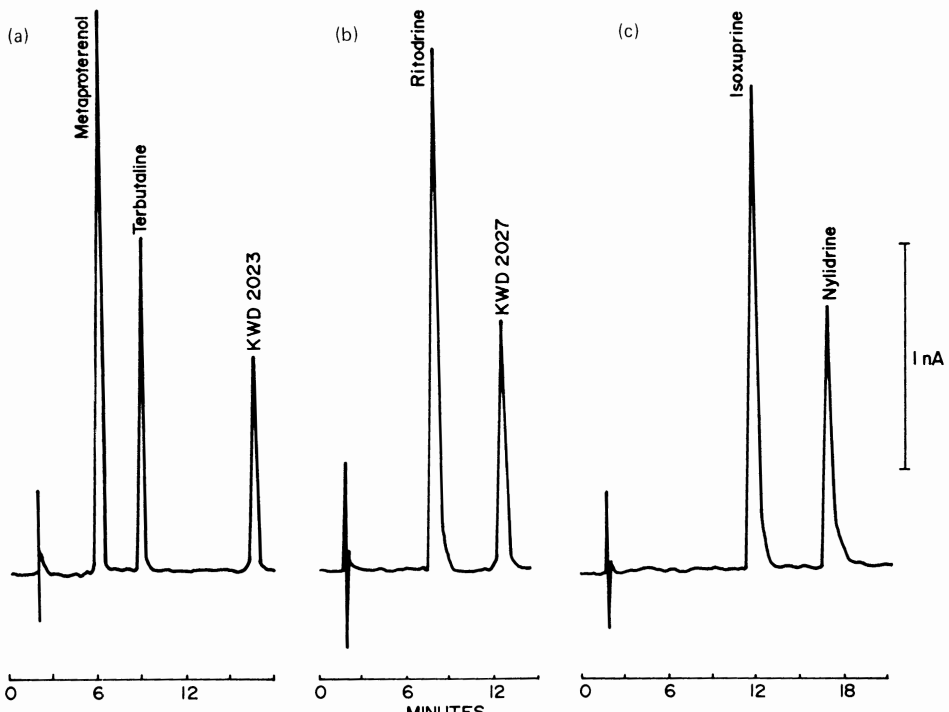

Figure 11 Beta mimetics. The chromatography was performed isocratically with a methanol:phosphate buffer mobile phase. The amount of methanol in the mobile phase depended on the polarity of the compounds of interest. (a) 15% MeOH in mobile phase, metaproterenol (10 ng), terbutaline (10 ng), KWD 2033, the internal standard (20 ng). (b) 25% meOH in mobile phase, ritodrine (10 ng), KWD 2027, the internal standard (20 ng), (c) 40% MeOH in mobile phase, isoxsuprine (20 ng), and nylidrin (20 ng). (Reproduced with permission of BAS Press.)

hypertensive drug methyldopa, has been measured both in the biological fluids and dosage forms. Detection limits are in the low nanogram per milliliter range.

Levodopa and carbidopa are used in combination to treat Parkinson's disease. Carbidopa is a dopa decarboxylase inhibitor which can significantly reduce the dosages of levodopa required for therapy.

Table 4 Beta Blockers and Beta Mimetics[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Naldolol	B	RP	GC	+1.30	100 pg	42,44
Sulfinalol HCl	B,U	NP	GC	+0.73	10 ng/mL	43
Mepindolol	B	RP	GC	+1.40	5 ng/mL	45
8-Hydroxycarteolol	B,U	IE	CP	+0.65	5 ng/mL	46
Ritodrine	S	IE	GC	+0.85	127 pg, 455 pg	52
Isoxsuprine						
Ritodrine	B	RP	GC	+0.90	0.2 ng	48,49
Isoxsuprine						
Metaproterenol						
Terbutaline						
Nylidrin						
Soterenol	DF	RP	CP	+1.0	10 ng/mL	50
Isoproterenol						
Salbutamol	B	IE	CP	+0.95 (SCE)	0.5 ng/mL	51
Fenoterol						
Terbutaline	B	RP	GC	+0.90	5 pmol/mL	47
SK&F 82526 (a benzazepine with a catechol moiety)	B	RP	GC	+0.65	50 pg/mL	54
Colterol	B,U	RP	GC	+0.60	5 ng/mL	53

[a]Abbreviations used: see Table 1.

Since the ratio of dopamine to L-dopa in the plasma indicates the effectiveness of the inhibitor, measurement of these compounds in body fluids is useful. Most methods rely on a simple alumina extraction of these compounds followed by LC-EC detection at an applied potential ranging from +0.70 V to +0.90 V (see Table 5).

F. Tricyclic Antidepressants

The tricyclic antidepressants used in the treatment of endogenous depression have been widely prescribed in recent years. The tetracyclic antidepressant mianserin is now undergoing clinical trials. Since there is a strong correlation between the plasma levels of these compounds and their therapeutic efficacy, there is a great demand for routine laboratory procedures to assay plasma samples for these drugs and their metabolites.

Laboratories performing therapeutic drug monitoring require techniques that are sensitive, selective, and simple. Present methodologies include GC-MS, LC-UV, and LC-EC. With GC-MS, assays are often long, requiring the synthesis of derivatives. LC-UV methodologies lack sensitivity; other methodologies cannot achieve the required detection limits and also have a problem with interfering endogenous compounds. LC-EC (Fig. 12) offers the advantage over GC procedures of measuring the compounds directly. Both the parent drug and the metabolites can be measured in a single chromatogram with LC-EC (Fig. 12). This is critical because some of these drugs produce active metabolites which should be monitored.

The standard protocol for assay of these compounds utilizes reversed-phase liquid chromatography with electrochemical detection at an applied potential of +1.05 V. The analysis of both the tricyclic antidepressants and the tetracyclic antidepressant, mianserin, potentially could both be performed on the same chromatographic system with only slight modifications in mobile phase. Detection limits for this methodology are in the low nanogram range (see Table 6).

G. Phenothiazines

Phenothiazines have been prescribed for the treatment of psychotic patients because of their production of mood and behavior improvement without causing addiction or excessive sedation. There is a strong correlation between the plasma concentrations of the phenothiazines and their therapeutic outcomes. However, their concentrations in plasma are quite low due to the low therapeutic dosages used, the extensive metabolism on oral administration, and a large distribution volume due to multiple-site binding. Phenothiazines are also somewhat unstable and can be partially lost during sample handling. Generally, concentrations are in nanograms per milliliter; therefore, highly sensitive techniques must be employed.

Table 5 L-Dopa and Related Compounds[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Levodopa Carbidopa Methyldopa Impurities	DF	RP	GC	+0.90	60-80 ng	60
Methyldopa	B	RP	GC	+0.75	NK	61
Levodopa Dopamine	B	IE	CP	+0.72	10 ng/mL	62
Methyldopa	B	RP	GC	+0.80	10 ng/mL	63
Carbidopa Levodopa DOPAC	B	RP	GC	+0.70	15 ng/mL	64
Methyldopa Methyldopate	B,M	IE	GC	+0.75	0.05 μg/mL	65

[a]Abbreviations used: see Table 1.

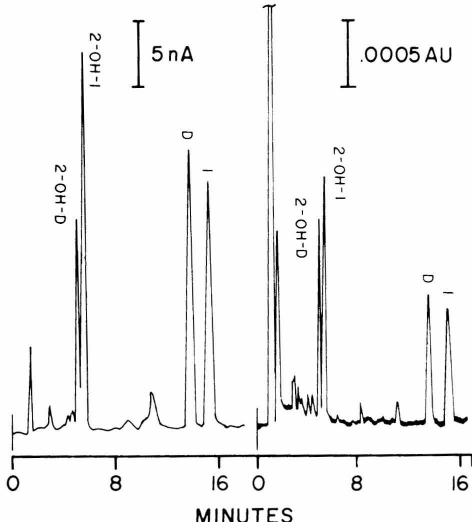

Figure 12 Tricyclic antidepressants in plasma. Comparison of spiked chromatograms obtained simultaneously by EC and UV detection. Legend: 2-OH-D = 2-hydroxydesipramine, 32 ng injected; 2-OH-1 = 2-hydroxyimipramine, 38 ng injected. As can be seen, EC detection has less baseline noise and a better signal response than UV detection. The applied potential is +0.05 V. (Reproduced with permission of Bioanalytical Systems, Inc.)

Methodologies that have evolved include GC, RIA, LC-UV, and LC-EC. GC-MS assays are long and tedious, requiring the synthesis of derivatives as well as expensive instrumentation. GLC and LC-UV methods have rather high detection limits. Biological assays such as RIA suffer a serious problem of cross-reactivity between the parent compounds and metabolites. Likewise, radioreceptor assays cannot distinguish between the drugs and metabolites and thus are, by definition, nonspecific. LC-EC methods have been developed which are rapid and have the sensitivity required to determine very low concentrations (Fig. 13). Also, it is well known that several drugs are simultaneously used in psychiatric practice, which makes the ability to monitor these compounds in the same analytical run highly desirable.

Curry et al. describe a method for concurrent monitoring of phenothiazine, thioxanthene, and butyrophenone neuroleptics using LC-EC [72,73]. As many as 21 compounds could be determined using this methodology. Other methods are designed to measure several phenothiazine compounds and metabolites simultaneously (see Table 7).

Table 6 Tricyclic Antidepressants[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Imipramine, desipramine, and metabolites	B	RP	GC	+1.05	5 ng/mL	55-57
Mianserin metabolites	B	RP	GC	+1.05	5 ng/mL	58,59

[a]Abbreviations used: see Table 1.

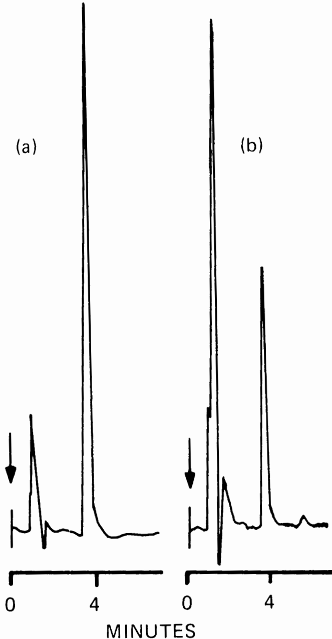

Figure 13 Chlorpromazine in plasma. Spiked plasma extract chromatograms. (a) 60 ng chlorpromazine injected, corresponds to 120 ng/mL plasma, 20 nAFS. (b) 7.2 ng chlorpromazine injected, corresponds to 14.4 ng/mL plasma, 5 nAFS. Samples were extracted with hexane and reconstituted in mobile phase (60% 0.2 M H_3PO_4, 40% MeOH, containing 2.5 mM tridecylamine, pH = 2.5). Chromatography was performed on a BAS Biophase Octyl 5-μm column with detection on a glassy carbon electrode set at an applied potential of +0.85 V. (Reproduced with permission of Bioanalytical Systems, Inc.)

H. Miscellaneous Drugs

1. Thiols and Disulfides

As described in Sec. II, there are some functional groups which are very uniquely suited to the LC-EC approach. Thiols (mercaptans, sulfhydryls) and the corresponding disulfides form some of the most important redox couples in biochemistry (e.g., glutathione and glutathione disulfide). There are relatively few drugs containing these groups; however, captopril and penicillamine have received considerable attention in recent years. Such compounds can be very difficult to deal with due to their instability in the presence of dissolved oxygen. This problem is more often responsible for establishing a methods detection limit than is the instrumentation itself. With proper

Table 7 Phenothiazines[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Phenothiazines	S	RP	CP	+1.2	NK	66
Prochlorperazine	P	RP	GC	+0.85	0.2 ng/mL	67
Trimeprazine	B	NP	GC	+0.90	0.5 ng/mL	68
Chloropromazine Levomepromazine	B,U	RP	GC	+0.95	100 pg	69
Promethazine and other phenothiazines	B	NP	GC	+0.90	0.2 μg/mL	70
Perphenazine Fluphenazine	B	RP	NK	NK	1 ng/mL	71
Phenothiazine, thioxanthene and butyrophenone Neuroleptics and antihistamines	B	NP	GC	+0.90, +1.1	0.01 ng/mL	72,73,76
Chlorpromazine	B	NP	GC	+0.85	1 ng	74,77
Chlorpromazine	B	NP	GC	+0.90	0.25 ng/mL	75,78

[a]Abbreviations used: see Table 1.

sample handling, LC-EC would appear to be the method of choice for these compounds (Table 8). In most cases, the drugs have been detected directly; however, the instability problem can be partially overcome by derivatization [84]. *N*-(4-dimethylaminophenyl)maleimide was coupled to the thiol (captopril) using the traditional 1,4-addition reaction, protecting the thiol from oxidation. The tertiary amine on the derivatizing agent is electrochemically oxidizable at a more positive potential than the thiol. In this approach, one sacrifices the selectivity inherent in the low thiol oxidation potential in order to avoid decomposition of the analyte. The development of analytical methods often involves such compromise. Direct detection of the thiol usually proves to be satisfactory. Mercury electrodes generally give the best detection limits and selectivity, but glassy carbon can also be used (see Table 9). Figure 14 depicts the simultaneous determination of captopril and its corresponding disulfide using a dual-series detector with mercury film electrodes.

2. *Nitrogen Heterocycles*

A large number of diverse pharmaceuticals fall into this broad classification. Many of them are electroactive and have fit the earlier sections of this review. Others are unique, both from pharmacological and electrochemical points of view. The calcium antagonist nifedipine is a good example. The heterocyclic ring can undergo

a two-electron oxidation (as per NADH), and the other ring bearing the nitro group can be easily reduced. Nifedepine and its metabolite therefore offer unique opportunities for LC-EC methods development, and work in this area is well under way.

Physostigmine is another unique structure which acts as a rather

Table 8 Thiol-Containing Compounds[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
D-Penicillamine	B	RP	GC	+0.80	5 ng	89
D-Penicillamine	B	RP	GC	+1.08/+1.20	10 ng	87
Penicillamine disulfide						
D-Penicillamine	B,U	IE	Au	+0.80	0.05 μg/mL	79
D-Penicillamine	B,U	IE	Hg	+0.10	10^{-7} M	80,85,86
D-Penicillamine	B,U	IE	Hg	+0.10	20 ng	81
Captopril	B	RP	Hg	+0.10	0.4 ng	82
Captopril	B,U	RP	Hg	+0.07	1 pmol	83
Captopril	B	RP	GC	+0.90	10 ng/mL	84
N-Acetylcysteine	B	RP	Hg	+0.10	1 pmol	88

[a]Abbreviations used: see Table 1.

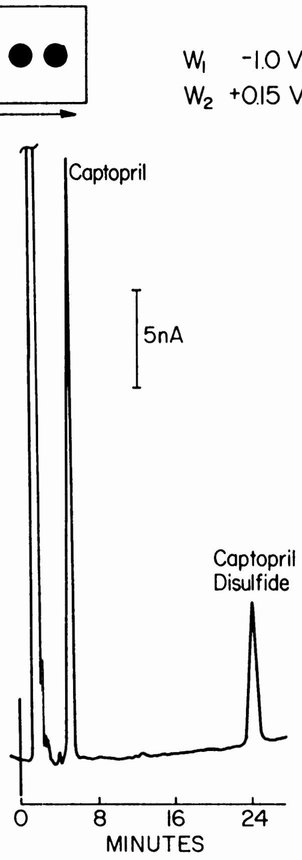

Figure 14 Captopril in urine. Dual Hg/Au chromatogram of acidified urine spiked with captopril (10 μg/mL) and captopril disulfide (20 μg/mL). The urine was prepared by adding 200 μL of 1 M $HClO_4$ containing 3 g/L Na_2EDTA to 1 mL of urine. The very clean appearance of the complex urine matrix is due to the high degree of selectivity inherent in the dual Hg/Au detector for thiols and disulfides. After being chromatographically resolved, these compounds pass into the dual detector where the disulfides are reduced at the upstream electrode, and the thiols are simply detected at the downstream electrode. The result is a single chromatogram depicting both thiols and disulfides. (Reproduced with permission of Bioanalytical Systems, Inc.)

strong cholinesterase inhibitor. The compound readily oxidizes electrochemically and can be detected at very low concentrations in biological fluids. Cimetidine (Tagamet) has become one of the most important drugs

in the last few years due to its effectiveness in the treatment of peptic ulcers. The electrochemistry of this compound is not well understood and the oxidation potential is quite high. It does not benefit from fused rings in providing a thermodynamic driving force as does physostigmine. Nevertheless, LC-EC has been useful for determination of this drug.

The antimalarial primaquine is an aromatic amine fused to a heterocyclic ring. The compound oxidizes at very favorable potentials at a graphite electrode (see Table 9).

The neutroleptic haloperidol is also electrochemically active, but at this point, the oxidation mechanism is totally unknown. Korpi et al. have

presented some excellent results on LC-EC determination of haloperidol and its reduced metabolite (keto group reduced to an alcohol) [92].

At least two research groups have investigated the LC-EC determination of methylxanthines (e.g., theophylline). While these methods have been successful and certainly have some merit for fundamental

Table 9 Miscellaneous Drugs[a]

Drug	Sample	LC mode	W	E (in volts, vs. Ag/AgCl)	MDA/MDC	Reference
Haloperidol	B	NP	GC	+0.90	0.5 ng	92
Primaquine	B,U	NP	PG	+0.30/+0.50	200 pg	93
4,5,6,7-Tetrahydroisoxazolo [5,4-c] pyridin-3-ol	S	RP	GC	+0.98/+0.95	50 pmol	94
Nifedipine	B	RP	GC	+0.98/+0.60	20 ng/mL	95,96
Physostigmine	B	NP	GC	+0.80	0.5 ng/mL	97
Cimetidine	B,U	RP	CP	+1.05	0.62 μg/mL	98
Lidocaine	B	NP	GC	+1.20	0.50 μg/mL	99
Theophylline	S	NP	CP	+1.24 (SCE)	0.5 mg/mL	90
Theophylline	S	RP	GC	+1.30 (SCE)	90 ng	91
Progabide	B	RP	GC	+1.0	30 ng/mL	100
Progabide acid					15 ng/mL	

[a]Abbreviations used: see Table 1.

studies, there are so many satisfactory alternative TDM procedures for theophylline that LC-EC is probably not needed.

3. *Aromatic Amines and Phenols*

We have already examined a number of drugs which fit either of these classes. A perusal of the *Merck Index* will reveal the staggering number of compounds in these two classes. And all of them can be determined by LC-EC! We leave the reader with two final examples: Lidocaine, a local anesthetic, and progabide, a GABA mimetic and anticonvulsant. As is usually the case, reversed-phase chromatography with

glassy carbon LC-EC detection does the job very nicely.

IV. FUTURE DIRECTIONS

A. Multiple-Electrode LC-EC

Electrochemistry in thin layers of solution is a very highly developed field of electroanalytical chemistry. The thin-layer geometry is ideal for LC-EC in that it provides a very rugged, low-volume transducer which can faithfully reproduce the shape of concentration profiles ("peaks") eluting from very efficient LC columns. Figure 3 illustrates the most popular LC-EC detector cell. The thin-layer channel is defined by a gasket held between the upper block (a stainless-steel auxiliary electrode with low dead-volume fittings) and the lower block (an inert polymeric material containing one or more working electrodes at which the reactions of interest occur). The effective dead volume can be made less than 1 μL, a very difficult task for optical detectors. The thin-layer cube is easily disassembled for cleaning and for changing working electrodes, an important advantage for routine work in a chemical laboratory. The simultaneous use of two or more working electrodes greatly improves both the qualitative and quantitative aspects of an LC-EC experiment. Electrodes of the same or different materials, shapes, and surface areas may be used, and the electrode potentials may be independently controlled.

In the "parallel mode" the compounds eluting from the column pass over each electrode at the same time. The following applications are quite useful:

1. The ratio of currents monitored at each electrode can provide confirmation of peak identity and purity.
2. Oxidations and reductions can be carried out simultaneously. This saves time and enhances selectivity. This can be ideal for compounds present in several different redox states.
3. Signals from low and high potential reactions can be recorded simultaneously, providing both greater selectivity and wider applicability in a single experiment.
4. A difference signal can be plotted to subtract out "common mode" information while enhancing detection of the desired compound.

In the "series mode," the lower block is rotated 90° in relation to the flow stream. Products of the upstream electrode reaction can be detected downstream. If an oxidation is carried out upstream, a reduction is accomplished downstream and vice versa. The following applications are popular:

1. The ratio of currents monitored at each electrode can provide confirmation of peak identity and purity.
2. Selectivity is enhanced at the downstream electrode because compounds with chemically irreversible reactions upstream are discriminated against.
3. The upstream electrode can "derivatize" compounds to enhance detectability at the downstream electrode. Overall selectivity and detection limits can be greatly improved.
4. Dissolved oxygen can be discriminated against, simplifying LC-EC of compounds that ordinarily would require mobile-phase deoxygenation (e.g., nitro compounds).
5. "Common mode" currents can be discriminated against by taking the difference between the two signals.

Both series and parallel dual-electrode LC-EC already provide many opportunities for study of drug metabolism because of the wide range of redox properties involved. This more recent development has many features to recommend it; however, the vast majority of LC-EC applications are quite adequately carried out using a single (less expensive) working electrode.

B. Precolumn Derivatization for LC-EC

Almost all tyrosine and tryptophan metabolites of neurochemical interest are electrochemically reactive and can be *directly* determined by LC-EC. Without a doubt, this fact is the primary reason the technique has become so popular in the last few years, in contrast to other

methodologies which require precolumn derivatization. Simple assays (few steps) always are the most reliable. Nevertheless, there are some problems in which derivatization appears to be the only practical possibility. Amino acids, with few exceptions, are universally determined by derivatization whether the technique be optical absorbance, fluorescence, gas chromatography, liquid chromatography, or thin-layer chromatography. Fatty acids (e.g., prostaglandins), alcohols, alkylamines, and many thiols also present a challenge for most analytical methods. This challenge is often met by derivatization.

A large number of classic (e.g., 2,4-dinitrophenylhydrazine) and more recent (e.g., 2,4,6-trinitrobenzenesulfonic acid) derivatizing reagents are based on the excellent optical properties of the aromatic nitro group. Fortunately, this same group is ideal as a derivatization approach for LC-EC. The nitro group is easily reduced to a hydroxylamine:

$$\phi NO_2 + 4e + 4H^+ \longrightarrow \phi NHOH + H_2O$$

Using a dual-series detector the latter can be oxidized to the nitroso derivative at very low positive potentials:

$$\phi NHOH \longrightarrow \phi NO + 2e + 2H^+$$

Because the nitro group is quite unusual in biology, it provides a very selective handle for LC-EC of many nonelectroactive compounds. Derivatives with more than one nitro group add sensitivity (more electrons transferred per molecule). Several good examples have appeared in the literature [101], including determination of GABA in brain tissue.

Recently, Joseph and Davies discovered that the *o*-phthalaldehyde/mercaptoethanol (OPA) derivatives of amines, commonly used for LCF of amino acids, are ideal for LC-EC as well [102]. The chemistry is illustrated in Fig. 15. The isoindole product is readily oxidized electrochemically, providing a very useful approach to a variety of alkylamines, amino acids, and small peptides. This discovery greatly expands the potential role of LC-EC in biomedical research. There is a strong possibility that OPA derivatives will be widely studied by LC-EC. The detector response is more uniform than for the LCF technique, where for certain compounds, detection limits suffer due to a poor fluorescence quantum yield.

CHO CHO + RSH —($R'NH_2$; 2 min., 20 °C)→ SR, N − R'

Figure 15 Derivation of primary amines to form electroactive isoindoles.

C. Postcolumn Reactions in LC-EC

Postcolumn chemical reactions coupled to electrode reactions are also expanding the range of LC-EC applications. Figure 16 illustrates four configurations. In (a), a reagent is added, mixed, and reacted in a delay line, followed by electrochemical detection. A superb example of this is the determination of acetylcholine in brain tissue by reversed-phase LC-EC [103]. Acetylcholinesterase and choline oxidase are mixed in, and the detection process proceeds as follows:

$$\text{acetylcholine} \longrightarrow \text{choline} + \text{acetic acid}$$

$$\text{choline} \longrightarrow \text{betaine} + H_2O + 2H_2O_2$$

The peroxide is detected electrochemically at a platinum electrode. There are many pairs of esterases and oxidases which have been used to develop classical assays. Now many of these can be directly coupled to LC-EC. In addition, a possibility now being explored involves the

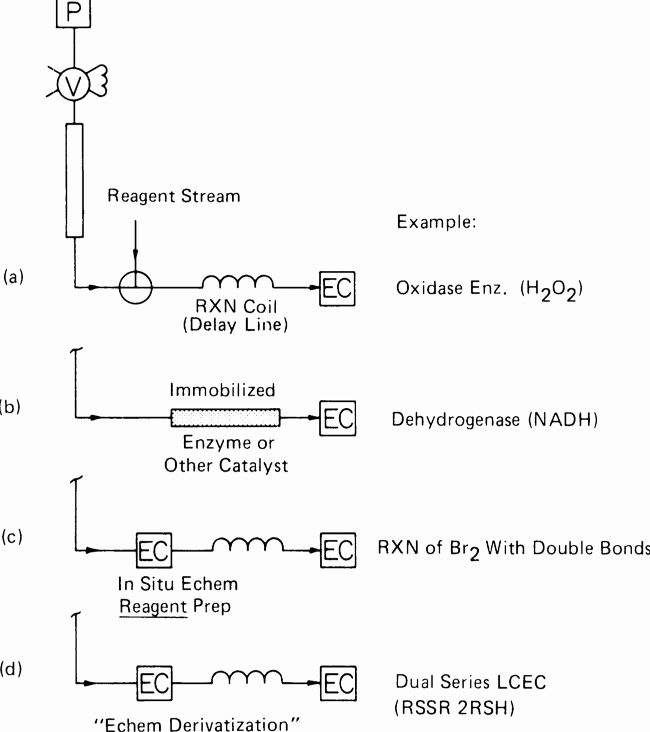

Figure 16 Configurations for post-column reactions with electrochemical analyzers. Various combinations of these four arrangements have also been used.

use of enzymes to hydrolyze drug conjugates prior to electrochemical detection of the free phenol.

In (b), a catalyst is immobilized and a cofactor is detected. For example, using a dehydrogenase enzyme, an alcohol can be detected indirectly by monitoring the turnover of NAD to NADH, the latter being ideal for electrochemical detection. In (c), an upstream electrode generates a reagent (e.g., Br_2 from Br^- in the mobile phase) which reacts with a nonelectroactive compound (e.g., an unsaturated fatty acid), and the *decrease* in reagent concentration is monitored downstream [104].

In (d), the analytes of interest are converted at an upstream electrode into a product which is more selectively detected downstream. Examples already mentioned include reduction of a nitro compound to a hydroxylamine and reduction of a disulfide to a thiol.

V. CONCLUSION

It was the intention of this brief overview to indicate that LC-EC technology is advancing rapidly. In the past few years, the technique has become quite reliable and now exhibits significantly better sensitivity and detection limits than in the early 70s. Novel electrode materials, pre- and postcolumn reactions, and multiple working electrodes have extended the quantitative aspects of LC-EC while dramatically increasing the range of applicable compounds. LC-EC is not going to be a workhorse of routine TDM in the same sense as LC-UV. Nevertheless, it is used for this purpose in many research laboratories where suitable drugs are under study.

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II

MAJOR CLASSES OF DRUGS

9

ANTIASTHMATICS

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I. INTRODUCTION

Bronchial asthma is a chronic disease characterized by episodic small airway obstruction that is clinically manifested by wheezing dyspnea, cough, and production of mucoid sputum. Although asthma can develop at all ages, approximately half of the cases occur before age 10. The incidence in the United States and other countries is about 3% of the population but is very low among American Indians, Eskimos, and West Africans.

Asthma, being an episodic disease, can manifest by acute attacks lasting minutes to hours interspersed among long, symptom-free periods. However, many patients experience some degree of persisting airway obstruction that requires the use of continuous medication to relieve the symptoms. The principal drugs used are bronchodilators, cromolyn sodium, and corticosteroids. The corticosteroids are best reserved for acute situations and are used chronically in only those patients with serious morbidity. Cromolyn sodium has prophylactic effectiveness in a limited population, and there are major compliance problems. In the bronchodilator class are three groups of drugs: (a) methylxanthines, primarily theophylline; (b) sympathomimetic drugs, especially the β_2-adrenergic agonists; and (c) anticholinergics. The latter group has had minimal therapeutic use primarily as adjuncts.

Therapeutically the sympathomimetic drugs are monitored by their physiological responses. In the United States in recent years, theophylline has become established as the primary drug for controlling

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chronic asthma symptoms in both children and adults; it or caffeine is also used in neonatal apnea. The optimal use of theophylline in asthma requires the individualization of the dosage regimen to maintain the serum concentration within the therapeutic range of 10-20 μg/ml. For treatment of neonatal apnea, most authorities recommend a desired concentration of 5-10 μg/ml. Therapeutic drug monitoring of theophylline has become common and routine. The emphasis of this chapter is to review the liquid chromatographic procedures in the English literature that have been developed for the determination of theophylline and related xanthines.

II. RATIONALE FOR XANTHINE MONITORING

Although theophylline was known to be of value in the clinical therapy of asthma since the beginning of this century, serious toxicity had restricted it value. A persistant use was in status asthmaticus, particularly when the patient was resistant to adrenaline therapy. In 1970, Maselli et al. [1] compared the efficacy of rapid versus slow infusion of theophylline. Forced expiratory volumes in the first second (FEV_1) were promptly improved by the rapid infusion (5 min) of theophylline but only slowly improved with slow infusion (8 hr). A bronchodilator effect was demonstrated at plasma theophylline levels of 3 μg/ml (17 μM), but more improvement was observed at higher doses.

Realizing the difficulty of measuring pulmonary function in acutely ill asthmatic patients, Mitenko and Ogilvie [2,3] established a clear log plasma theophylline concentration-response relationship in asthmatic adult patients between attacks. Incremental steady-state plateau plasma theophylline concentrations between 5 and 20 μg/ml (28-110 μM) plotted logarithmically were associated with a linear improvement in FEV_1. The steady-state theophylline concentration requirement is important in defining this relationship since the site of bronchodilator activity of theophylline is in the peripheral kinetic compartment [4,5].

The association between theophylline concentrations and efficacy or toxicity has been established by numerous studies (see [6-10]). A safe upper limit of serum theophylline concentration fo 20 μg/ml (110 μM) has been clearly defined; the lower limit is a softer number, between 3 and 10 μg/ml (17-55 μM). Only an average of 40% of the total possible improvement in FEV_1 in acute asthma has been achieved at a theophylline concentration of 20 μg/m; [1,3,11-12]. Theophylline has a major effect only on smooth muscle contraction and not on the two other factors of mucosal thickening and mucus plugging, each of which contributes to the airway obstruction in asthma. It is thus important to monitor theophylline concentrations, as complete resolution cannot always be achieved by just increasing the dosage. More importantly, the minor adverse effects of nausea, vomiting, headaches, and irritability do not necessarily precede the life-threatening toxicity of cardiac

arrhythmias, seizure, and/or death [8,13-14]. The incidence of toxicity increases progressively as theophylline concentrations exceed 20 μg/ml.

Hendeles, Weinberger, and co-workers have published extensively on guidelines for achieving and maintaining appropriate theophylline concentrations (see [6,15-16]). An initial loading dose of 5 mg/kg can be used if no theophylline has been taken in the previous 24 hr. In emergencies, 2.5 mg/kg is given if an immediate serum measurement is unavailable. On average, for every 1 mg/kg of theophylline administered, the serum concentration will increase by 2 μg/ml. Two tables are appropriate for inclusion in this chapter. Table 1 lists recommendations to achieve a target serum theophylline concentration of 10 μg/ml according to patient/risk factor.

Some patient/risk factors are the result of changes in theophylline clearance caused primarily by differences in rate of hepatic biotransformation [17-26]. Besides those listed in the table, two other factors should be mentioned. High carbohydrate, low protein diets and consumption of dietary methylxanthines will decrease theophylline elimination; in converse, a low carbohydrate, high protein diet will increase elimination [27-28]. Charcoal-broiled meats can also increase elimination. Concurrent administration of drugs such as macrolide antibiotics [29-30] and cimetidine [31] will reduce theophylline clearance.

Table 2 lists recommendations for adjustment in dosage once steady-state theophylline serum concentration has been determined.

The other respiratory disease where theophylline or caffeine is routinely used is in the treatment of neonatal apnea [32-37]. Premature newborn infants treated with theophylline produce significant plasma concentrations of caffeine. This is the result of the marked deficiency of clearance of caffeine in the neonate [38-41]. Therapeutically this means that both theophylline and caffeine need to be monitored, and either one can be used successfully as the therapeutic agent.

III. LIQUID CHROMATOGRAPHY

A. Theophylline

The measurement of theophylline concentrations in serum by liquid chromatography has been a relatively straightforward analytical problem. With slight modifications, most standard chromatographic conditions are suitable. The first groups to publish a theophylline assay were Manion et al. [42] using liquid-solid chromatography, oxypropionitrile bonded on Porasil C, with hexane/isopropanol gradient elution at 37°C, and Thompson et al. [43] using ion-exchange chromatography, Aminex A-5 cation resin with 0.45 M ammonium phosphate buffer at pH 3.65 as eluent. The Weinberger and Chidsey [44] modification of the Thompson et al. procedure was the first LC procedure to have extensive clinical use. The Orcutt et al. [45] procedure was widely popularized. Their

Table 1 Chronic Dosing Guidelines for Total Daily Dose of Theophylline Before Serum Concentration Measurements

Patient/risk factor	Age	mg/kg/day[a,b,c]
Neonates	Postnatal age up to 24 days	2
Neonates	Postnatal age beyond 24 days	3
Infants	6-52 weeks old	mg/kg/d = (0.2) × (age in weeks) + 5.0
Children	1-9 years old	20
Older children	9-12 years old	16
Adolescents (cigarette or marijuana smokers)	12-16 years old	16
Adolescents (nonsmokers)	12-16 years old	13
Adults (otherwise healthy cigarette or marijuana smokers)	16-50 years old	13
Adults (otherwise healthy nonsmokers)	>16 years old (including elderly	10 (not to exceed 900 mg/day
Cardiac decompendation, cor pulmonale, and/or liver dysfunction		5 (not to exceed 400 mg/day

[a]These recommendations, based upon mean clearance rates for age or risk factor, were calculated to achieve a target concentration of 10 μg/ml, except for neonates, where the target concentration for neonatal apnea is 7.5 μg/ml.
[b]When administered as a constant infusion, divide dose by 24 for hourly rate; 80% aminophylline = theophylline dose. In patients with less severe symptoms, a rapidly absorbed oral formulation (solution, chewable tablet, or daily dose is divided into equal parts and administered at 4-hr intervals for children and 6-hr intervals for infants and adults.
[c]Use ideal body weight of obese patients.
Source: L. Hendeles, University of Florida Drug Therapy Service.

Table 2 Final Dose Adjustment Guided by Measurement of Serum Theophylline Concentration

Peak theophylline level, μg/ml	Approximate adjustment in total daily dose	Comment
<5	100% increase[a]	If patient is asymptomatic, consider trial off drug, repeat measurement of serum concentration after dose adjustment
5-7.5	50% increase	
8-10	20% increase	Even if patient is asymptomatic at this level, an increased serum concentration may prevent symptoms during a viral upper respiratory infection (URI), or heavy exposure to an inhalent allergen or vigorous exertion
11-13	Cautious 10% increase if clinically indicated	If patient is asymptomatic, no increase is necessary, if symptoms occur during URI or exercise, increase as indicated
14-20	None	If "breakthrough" in asthmatic symptoms occur at the end of dosing interval, change to sustained-release product and repeat serum level measurement
	Occasional intolerance requires a 10% decrease	If side effects occur, decrease total daily dose as indicated
21-25	10% decrease	Even if side effects are absent
26-30	25% decrease	Even if side effects are absent, omit next dose and decrease total daily dose as indicated, repeat measurement of serum concentration
31-35	33% decrease	
⩾35	50% decrease	Omit next two doses, decrease as indicated, and repeat measurement of serum concentration

[a]To avoid potential toxic reaction: (1) assure that the sample represents a peak level obtained at steady state (e.g., no missed or extra doses with close approximation of prescribed dosing intervals during previous 48 hr.); (2) repeat laboratory determination if not initially performed in duplicate; (3) the increase of 50% or 100% should be made in 25% increments at 2-day intervals to further assure safety and tolerance.

Source: Ref. 15.

protocol is shown in Table 3 with typical chromatograms in Fig. 1. This procedure was developed for an office practice. Finger-puncture blood samples were collected; the total time required for a single determination was about 25 min and less than 10 min of technician time. Having a reliable theophylline assay, the initial marketing goal was to provide the analytical capability to every physician that treated asthmatic patients.

The initial modifications of the procedure were some convenient simplifications, such as the use of standard volumes (100 μl for the patient sample and internal standards), and use of 20 μg/ml of β-hydroxyethyltheophylline rather than 36.7 μg/ml; the latter was selected initally to give a peak height equal to that of 20 μg/ml of theophylline so that at a glance, the technician could have determined whether the

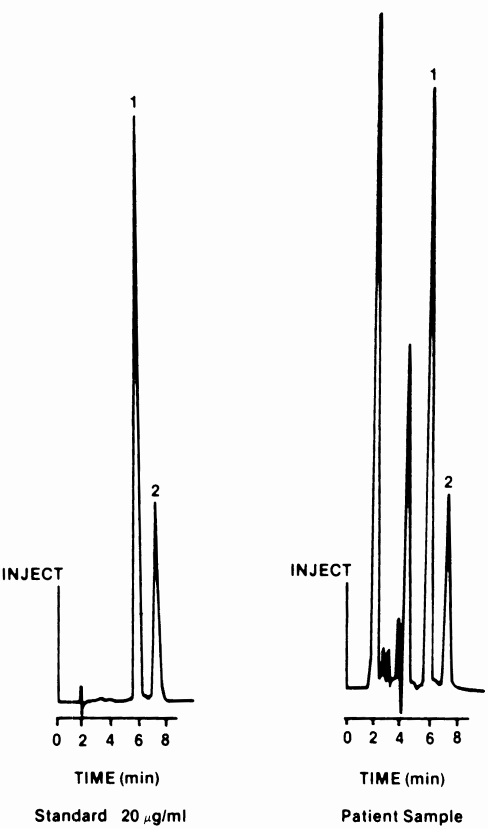

Figure 1 Typical chromatograms using a slight modification of the Orcutt et al. procedure. 1, theophylline; 2, β-hydroxyethyltheophylline (βHET).

Table 3 Theophylline Assay Procedure

Sample preparation:

1. Add 30 μl of internal standard solution (β-hydroxyethyltheophylline 36.7 μg/ml in 0.02 M sodium acetate, pH 4.0, 14% acetonitrile) and 30 μl of sample plasma/serum calibrator or control plasma to disposable test tubes.
2. Mix, then transfer to capillary tubes.
3. Centrifuge for 10 min at 13,000 rpm.
4. Inject 5-10 μl of supernatant.

Liquid chromatographic conditions:

Column:	μBondapak C_{18} (4 mm × 30 cm)
Temperature:	Ambient
Mobile phase:	0.01 M Sodium acetate, 7% acetonitrile, pH 4.0
Flow-rate:	2.0 ml/min
Pressure:	1500-2000 psi
Chart speed:	0.25 cm/min
Detector:	UV 254 nm
Full-scale absorbance:	0.01
Chromatography time:	8 min

Calculations:

1. Determine peak height for theophylline (T) and internal standard (I.S.).
2. Calculate the T/I.S. peak height ratio for each sample and the calibrator sample.
3. $\text{Theophylline } (\mu g/ml) = \frac{\text{T/I.S. (sample)}}{\text{T/I.S. (calibrator)}} \times \text{calibrator } (\mu g/ml)$
4. Periodic verification with standard curves.

Source: Ref. 45.

patient was at a toxic level. Rather than test tubes and capillary tubes, microcentrifuge tubes were vortexed and centrifuged for 2 min in a microfuge or equivalent centrifuge to decrease the total time requirements [46].

An alternative protocol for theophylline measurement is the "selected method of clinical chemistry" procedure of Broussard [47] which is a modification of Adams et al. procedure [48]. This procedure calls for an isopropanol/chloroform extraction of acidified serum or plasma, 8-chlorotheophylline as the internal standard, a mobile phase of 18% methanol, 1.6% acetonitrile, and 0.016% glacial acetic acid, and separation on a Spherisorb ODS column.

The alternative procedures proposed in the literature (Table 4) involve changes in any of the following: sample preparation, internal standard, mobile phase, column, or in the use of detectors. The sen-

Table 4 Clinical Theophylline Liquid Chromatography Assay Properties

Sample preparation	I.S.[a]	Mobile phase	Flow-rate (ml/min)	Column	Detector (nm UV)	Reference
A. Protein precipitation						
Acetonitrile	βHET	7% acetonitrile in 0.01 M sodium acetate, pH 4.0	2.0	μBondapak C_{18}	254	45
Acetonitrile	βHET	"	2.0	μBondapak C_{18}	254	99,105
Acetonitrile	βHET	"	2.0	μBondapak C_{18}	254,280	46
Acetonitrile	βHET	"	2.0	μBondapak C_{18}	275	100
Acetonitrile	βHET	"	2.0	μBondapak C_{18}	280	107
Acetonitrile	8CT	10% acetonitrile in 0.01 M sodium acetate, pH 4.0	2.0	Partisil 10 ODS	280	55,98
Acetonitrile	8CT	10% acetonitrile/KH_2PO_4	1.33	Partisil 10 ODS	280	57
Acetonitrile	8CT	5% acetonitrile, 0.05 M KH_2PO_4	3.0	μBondapak C_{18} 50°C	254	67
Acetonitrile	—	10% acetonitrile	2.0	ODS HC Sil-X-1	275	64
Acetonitrile	—	6% acetonitrile	3.0	μBondapak C_{18}	275	64
Acetonitrile	—	20% ethanol	1.5	PSX 10/25 ODS	254	111
Acetonitrile	βHET	4% methanol, 1% tetrahydrofuran, 0.01 M sodium acetate, pH 5.0	2.0	μBondapak C_{18}	280	92

Acetonitrile	βHET	6% methanol, 1.2% tetrahydrofuran, 0.01 M sodium acetate, pH 5.0	3.0	Radial Pak A	254,280	110
Acetonitrile	βHET	7% methanol, 1% tetrahydrofuran, 0.01 M sodium acetate, pH 5.0	1.5	Partisil PXS 5/25 ODS	254,280	110
Acetonitrile	8CT	20% methanol, 0.02 M sodium acetate, pH 5.5	2.0	Lichrosorb RP-8 (Vydac ODS precolumn)	UV	104
Methanol	βHET	10% acetonitrile in 0.01 M sodium acetate	3.0	Radial Pak C_{18}	280	96
Methanol	βHET	15% methanol in 0.02 M sodium acetate, pH 3.5	2.0	μBondapak C_{18}	273,254	101
Trichloroacetic acid, 4%	βHET	6% acetonitrile in 0.01 M sodium acetate, pH 4.0	2.0	μBondapak C_{18}	274	85
Trichloroacetic acid, 1.0 M	—	6.5% acetonitrile in 0.01 M sodium acetate, pH 4.0	3.0	Partisil 10 ODS	UV	69
Trichloroacetic acid, 5%	8CT	30% methanol in 0.02 M potassium chloride, pH 2.0	1.6	μBondapak C_{18}	280,254	84
Trichloroacetic acid, 9%	HPT	0.66% acetic acid		Zipax SCX	254,280	125

Table 4 (Continued)

Sample preparation	I.S.[a]	Mobile phase	Flow-rate (ml/min)	Column	Detector (nm UV)	Reference
Perchloric acid-potassium carbonate	—	25% methanol in 0.01 M sodium dihydrogen phosphate	0.8	μBondapak C_{18}	280	59
B. Extraction						
Isopropanol/methanol (1:1)	8CT	18% methanol 1.6 % acetonitrile 0.016% glacial acetic acid	1.5	Spherisorb ODS μBondapak C_{18}	273,280,254 254, 280	47
Chloroform/isopropanol (1:1)	8CT	2% acetonitrile in 0.01% acetic acid	1.5	ODS-Sil-X-1 55°C	273	48
Chloroform/isopropanol (1:1)	8CT	33% acetonitrile in 0.005 M aqueous acetic acid	1.0	Lichrosorb RP-8 55°C	273	103
Chloroform/isopropanal (1:1)	8CT	7.5% acetonitrile in 0.02% glacial acetic acid	1.0	Lichrosorb RP-8 55°C	273	129
Chloroform/isopropanol (1:1)	—	10% methanol 1% tetrabutylammonium chloride, 0.05 M sodium acetate, pH 4.2	1.0	Spherisorb ODS 50°C	280	91

Chloroform/isopropanol (19:1)	βHET	10% acetonitrile in 0.02 M sodium acetate, pH 4.0	1.8	μBondapak C_{18}	254	58
Chloroform/isopropanol (19:1)	—	chloroform/isopropanol/acetic acid, 84/15/1 (v/v)	0.7	Micropak SI 10	273	50
Chloroform/isopropanol (19:1)	βHET	8% acetonitrile in 0.02 M sodium acetate, pH 4.0	1.5	ODS-Hypersil	273	97
Chloroform/isopropanol (19:1)	8CT	74% methanol in 0.05 M $NH_4H_2PO_4$, pH 5.12	0.9	μBondapak	280	66
Chloroform/isopropanol (19:1)	HPT	0.66% acetic acid		Zipax SCX	254, 280	125
Chloroform/isopropanol (19:1)	IMX	chloroform, dioxane, formic acid, 95.5:4.5:0.01	1.4	RSL-RSil	273	93
Chloroform/isopropanol (19:1)	TB	chloroform/isopropanol/acetic acid (96:2:2)	2.0	Lichrosorb Si-60	275	79
Chloroform/isopropanol (19:1)	O	hexane to isopropanol gradient, 14-22% at 0.6%/min	1.8	Porasil C 37°C	270	42
Chloroform/isopropanol (20:1)	βHET	6% ethanol: 94% chloroform/hexane/acetic acid, 3/2/0.004	0.4	Zorbax SIL	254	53

Table 4 (Continued)

Sample preparation	I.S.[a]	Mobile phase	Flow-rate (ml/min)	Column	Detector (nm UV)	Reference
Chloroform/ isopropanol (20:1)	HPT	chloroform/*n*-heptane/ methanol, 39/56/5 (v/v)	3.0	Partisil 10	254	82
Chloroform/ isopropanol (9:1)	βHET	8% acetonitrile, 0.003 M sodium acetate, pH 4.5	2.0	μBondapak C_{18}	254	106
Chloroform/ isopropanol (3:1)	[βHET]	15% methanol in buffer, pH 6.6		FAST-LC-8	270	112
Isopropanol, chloroform, glacial acetic acid (5:4:1)	βHET	4% isopropanol in 0.01 M KH_2PO_4, pH 3.8	1.0	μMCH-10	277	108
Chloroform/ hexane (7:3)	8CT	*n*-hexane-ethanol, 76:24	1.5	μPorasil	280	70
Chloroform	8CT	18% methanol, 1.6% acetonitrile, 0.016% glacial acetic acid	1.4	μBondapak C_{18}	280	94
Chloroform	8CT	20% methanol in 1% propionic acid, pH 5.0	1.7	μBondapak C_{18}	280	61

Chloroform	8CT	25% methanol in 0.001 M sodium acetate, pH 4.0		Spherisorb ODS	280	56
t-Pentanol/ chloroform/ 0.1% HCl (80:19.9: 0.1)	—	hexane/isopropanol/water (80:19:1)	2.0	Partisil 5	280	63
Dichloro- methane	8CT	12% acetonitrile, 0.01 M sodium acetate, pH 4.0	2.0	S5 ODS	276	80
Dichloro- methane/ isopropanol (4:1)	βHET	7% acetonitrile, 0.01 M sodium acetate, pH 4.0	2.0	μBondapak C_{18}	254,280	102
Ethylacetate / acetonitrile (10:1)	βHET	9% acetonitrile, 0.07% tri- ethylamine and 0.055% acetic acid, pH 4.8	2.5	μBondapak C_{18}	204	71
Ethylacetate	—	0.66% acetic acid	1.0	Partisil SCX 50°C	275	95
Methylene chloride/ methanol (9:1)	HPT	20% methanol	0.75	MPLC RP-18	280	73
tert-Amyl alcohol, ethylene di- chloride (1:2)	βHET	10% acetonitrile in 0.026 M sodium acetate, pH 4.0	1.5	μBondapak C_{18}	254	65

Table 4 (Continued)

Sample preparation	I.S.[a]	Mobile phase	Flow-rate (ml/min)	Column	Detector (nm UV)	Reference
2-butanol, *n*-hexane (4:6)	—	1% H_2O, 5% methanol, 25% 2-butanol in *n*-heptane	3.33	μPorasil	280	54
C. Other						
Saline	8CT	0.45 M ammonium phosphate, pH 3.65	0.4	Aminex A-5 55°C	254	44,128
Ultra-filtrate	—	10% acetonitrile in 0.01 M sodium acetate, pH 4	2.0	μBondapak C_{18}	254	52
Ultra-filtrate	D	12% methanol in 0.05 M KH_2PO_4, pH 4.7	1.1	μBondapak C_{18}	254	60
—	—	35% methanol in 0.025 M potassium phosphate, pH 2.5	1.5	Partisil 10 ODS-2	254	62
—	βHET		1.0	μBondapak C_{18}	280	51

[a]Abbreviations: I.S. = internal standard; — = none, βHET = β-hydroxyethyltheophylline; 8CT = 8-chlorotheophylline; HPT = hydroxypropyltheophylline; IMX = 3-isobutyl-1-methylxanthine; D = dyphylline; O = oxazepam; TB = theobromine.

sitivity, precision, and accuracy of these procedures are sufficient for routine monitoring of theophylline concentrations in patients. In several papers, reliability was established on 1000-7000 patient samples. Vandemark [49], in his review of LC procedures for determining therapeutic theophylline levels, included a slightly different set of properties from those in Table 4.

Besides the chromatographic mode, a major distinguishing characteristic among the theophylline LC procedures is the method of sample preparation. A review of the data reported by the College of American Pathologists (CAP) therapeutic drug monitoring survey indicates that in 1981-1982, clinical laboratories were divided with regard to method of sample preparation: 40% protein precipition, 40% extraction, 15% direct injection [44], and 5% other (probably ultrafiltration [52,60]). Although the precision of liquid chromatographic procedures with solvent extraction is usually poorer than for direct LC analysis [72], the CAP data would indicate that when the procedures become routine in clinical laboratories, the various classes of procedures are equivalent with slightly less than 10% coefficient of variation for reference samples.

A synopsis of the many published LC procedures for theophylline is given in Table 4. In clinical laboratories, further modification occur. Some of these changes could be crucial. The concentration of acetonitrile or methanol used for protein precipitation, for instance, affects not only the extent of protein preciptition but also the solubility of drugs. Even using the same extraction solvent, the initial acidification, particularly when 8-chlorotheophylline is the internal standard, can change recoveries [56]. The basic procedures are suitable for either serum or plasma samples; however, the anticoagulant can cause problems. Nelson et al. [57] found that citrated plasma was unsatisfactory with their procedure because of a large peak that eluted with theophylline. In another procedure, citrated plasma can be used [44]. When the anticoagulant was the lithium or sodium salts of heparin or ethylenediaminetetraacetate, there were no problems [57]. In a third procedure, both citrate and ethylenediaminetetraacetate caused extensive tailing [111]. One source of ghost peak can be the parabens, common preservatives which have a long retention when being eluted with sodium acetate buffer on the reversed-phase columns [78]. Lipemic, iceteric, or hemolyzed sera can be used since they do not cause interferences [44,46]. Many of the standard analytical columns have been used for resolution. Balkon [73] reported that a medium-performance liquid chromatographic column, the MPLC RP-18 short Brownlee column, gave adequate resolution; thus a high-resolution analytical column is not critical.

In all of the commonly used clinical procedures, a UV spectrophotometric detector has been used at wavelengths of 254, 270-277, or 280 nm. The absorbance maximum for theophylline is 273 nm, but adequate

sensitivity is obtained at either 254 or 280, the wavelengths of the fixed-wavelength detectors. One difference between procedures is the use of single- compared to dual-wavelength detection. The other detector that could be used is the electrochemical detector (see Chaps. 3 and 8). Hansen and Dryhurst [74] were the first group to study the electrochemical properties of xanthines. Feasibility studies have been reported by Greenberg and Mayer [75], Lewis and Johnson [76], and Christensen and Isernhagen [77].

B. Caffeine and Theophylline Analogues

In all of the reversed-phase chromatographic procedures for the measurement of theophylline, caffeine will also be resolved [77-80] (see Fig. 2).

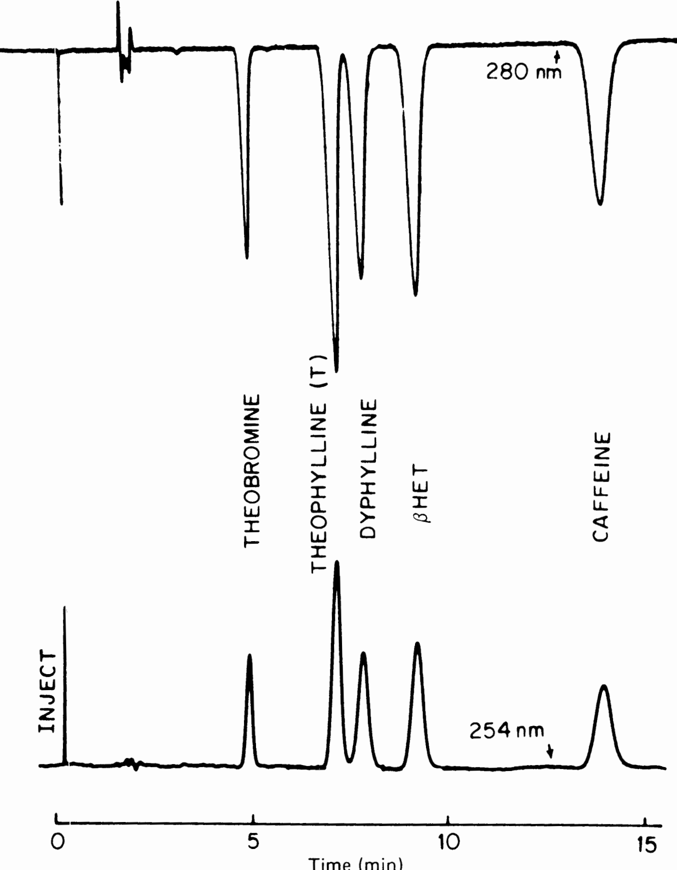

Figure 2 Chromatograms of standards. (From Ref. 46.)

Since caffeine-containing products are widely consumed by all population classes, a low serum concentration of 0.1-5 μg/ml (average, less than 1 μg/ml) should be anticipated. One study conducted in England of 600 random adult outpatients found that 5% of subjects had caffeine plasma concentrations in excess of 5.6 μg/ml [81].

Several *N*-7 substituted theophylline derivatives have been introduced into medicine with subsequent development of liquid chromatographic methods for quantification in serum [82-88]. These include dyphylline [7-(2,3-dihydroxyproprypyl)theophylline], proxyphylline-[7-(β-hydroxypropyltheophylline], acephylline (1,3-dimethylxanthine-7-acetic acid), and etofylline (β-hydroxyethyltheophylline). Dyphylline is the only one used in the United States. Its rapid clearance (2 hr half-life) has limited its use in chronic therapy [89-90]. Dyphylline separation from theophylline and caffeine is also illustrated in Fig. 2. Dungan et al. [46] suggest the weekly injection of a theophylline-dyphylline mixture as a better test to monitor column efficiency than a theoretical plate count determination. Since etofylline is β-hydroxyethyltheophylline, the most commonly used internal standard, an easy assay procedure would be to use a standard procedure with another internal standard such as dyphylline or 8-chlorotheophylline. Zuidema et al. [87-88] in their etophylline and acephylline procedure used the same sample preparation of adding 50 μl of 70% perchloric acid to 0.5 ml serum sample to denature and precipitate proteins, followed by centrifugation. Saturated potassium carbonate (50 μl) was added to return the pH to about 7.5, and the potassium perchlorate precipitate was removed with centrifugation. Chromatographic conditions for etophylline consisted of μBondapak C_{18} column, 5% acetonitrile in 0.01 M sodium phosphate (pH 6.2), at a flow-rate of 2.0 ml/min. No internal standard was used.

Theophylline, caffeine, and all of the *N*-7 substituted analogues can be determined by the same general LC methodology.

IV. INTERFERENCES

A. Paraxanthine

Orcutt et al. [45] initially reported a small interference, average of 0.25 μg/ml equivalent of theophylline, in about a quarter of their samples. A decrease of the acetonitrile concentration from 7% to 5% resolved this interference from theophylline. Acetaminophen is a likely candidate for this interference [71]. Furthermore, when high caffeine values were observed, an additional positive interference peak occurred which they presumed was paraxanthine, the major caffeine metabolite in human serum. Finally, in the first 2000 determinations of samples from asthmatic patients, Orcutt et al. had found no interference from other drugs.

Paraxanthine is not resolved from theophylline when reversed-phase columns are used in their normal mode. Even in this mode, the separation is a mixed retention mechanism rather than pure reversed-phase [61]. If dual detectors are used to determine the 280/254 ratio, both the unknown interferent, 0.36-0.48, and paraxanthine, 1.13, are readily distinguished from theophylline, 1.69 [46,78]. In a mixture of theophylline with paraxanthine, a linear relationship occurs which can be expressed as Y (% theophylline) = 192.4 × (280/254 ratio) − 216.6, $r = 0.99$ [78]. Paraxanthine can be separated from theophylline by using either normal-phase partition chromatography [78,93], ion-exchange chromatography [43,125] or with paired ion chromatography with a reversed-phase column [68,71,78,91]. Acetonitrile (4-5%) with "PIC A" (Water Associates) which contains 0.005 M tetrabutylammonium phosphate is one appropriate mobile phase.

Even if paraxanthine is not resolved from theophylline, this will not be a major source of error for most clinical samples. Paraxanthine is eliminated by humans at a rate similar to that for caffeine. Thus, depending upon when caffeine was consumed, the maximum paraxanthine concentration would approach that of caffeine, but more typically would be only 0.25-0.5 that of caffeine.

The highest reported elevation of serum theophylline values caused by paraxanthine is 3 μg/ml [45,92,110]. From a clinical management point of view, only one parameter would be affected, resulting in a lowering of the maximal effective concentration of theophylline from 20 μg/ml to 17 μg/ml. Since caffeine and most probably paraxanthine have some bronchodilator efficacy, the net loss is not even that great. Thus in actual practice, unless the caffeine concentration is inordinately large, the paraxanthine increase in the measured theophylline level would not affect the dosage for an asthmatic patient.

B. Medicinals

Leslie and Miller [94] reported that using an ethyl acetate extraction procedure [95], μBondapak C_{18} column, 1.4 ml/min flow-rate, 0.01 M acetate buffer, pH 4.0, containg 28% methanol, occasional salicylic acid interference occurred with older columns. This potential interference was eliminated by changing the mobile phase to 18% methanol, 1.6% acetonitrile, and 0.016% glacial acetic acid with a flow-rate of 2.0 ml/min. However, they found that with the new mobile phase, spuriously high results were obtained due to traces of ethyl acetate remaining in solution after the extraction. A satisfactory assay resulted when chloroform replaced ethyl acetate in the extraction procedure. Their results illustrate the potential problems with interference that can occur. Jowett [96] reported that the use of acetonitrile for protein serum precipitation caused a problem when a radially compressed C_{18} column (Radial Pak C-18) rather than a μBondapak C_{18} column was used. The use of methanol instead of acetonitrile overcome the peak

distortion. Naish and Cooke [97], using a ODS-Hypersil column, also reported an acetonitrile precipitation problem. Their solution was to extract the samples with isopropanol/chloroform. These results emphasize the need for verification of the procedure when different octadecylsilane (ODS) columns are used.

Many method papers include lists of various number of the drugs that do not interfere with the assay. As an example, in the Broussard paper [47], 80 specific drugs are named. Broussard states of his procedure that only salicylate (retention time, 3.1 min) could be a potent interferent of theophylline (retention time, 4.3 min) if it occurred at toxic levels and was analyzed on a column with poor resolution. Furthermore, monitoring a 254 would remove this potential. The evaluator of the Broussard procedure found the same results using the μBondapak C_{18} rather than the Spherisorb ODS column.

Besides salicylate, a few other medicinal compounds have been reported to interfere with some LC procedures for the measurement of theophylline. Using the nonextractive procedure of Franconi et al. [52], Soldin and Hill [58] found that two frequently used antibiotics, ampicillin and methicillin, had retention times close to theophylline. The use of a chloroform:isopropanol extraction procedure eliminated this interference. Other antibiotics reported to interfere with the analysis of theophylline by liquid chromatography include the cephalosporins [98-100,108] and sulfonamides [69,95,102-104,108]. Kelly et al. [98] reported that without extraction, two cephalosporin antibiotics, cefazolin and cephalothin, had retention times smaller to theophylline and their internal standard, 8-chlorotheophylline. Robinson et al. [99] reported that cephalothin (text), cephapirin (table and figure) interfered with the internal standard, β-hydroxyethyltheophylline, in their protein precipitation procedure. According to their table, both cephalothin and cefazolin had much longer retentions than theophylline and the internal standard. Agdeppa and Lipton [100] investigated nine cephalosporins using the Orcutt procedure [45]. They found only cepholexin as an interference to theophylline with good resolution of the other eight, including cefazolin, cephalothin, and cephapirin. Butrimovitz and Raisys [101], also seeking a time-saving, single-step protein precipitation method, reinvestigated the requirement for an extraction step for these antibiotics. They claim that samples deproteinized with two volumes of methanol containing β-hydroxyethyltheophylline as an internal standard and chromatographed with 15:85 methanol/sodium acetate buffer will give complete resolution. They calculated the theophylline concentration from the peak height at 273 nM but also monitor at 254 nM for possible chemical interferences. For theophylline, the 1-99th percentile ratio limit were 1:02 and 1:22; for β-hydroxyethyltheophylline (βHET) they were 0.95 and 1.01. The ratio limit for theophylline ratio/βHET ratio were 1.01 and 1.23.

Weidner et al. [108] in their routine procedure found interference in 3.8% of specimens. The interfering substances included cephazolin,

chloramphenicol, sulfamethoxazole, and sulfisoxazole. Their paper describes recognition, resolution, and a calculation technique for a reliable and clinically useful estimation of theophylline concentration when resolution was not achieved.

The resolution of the sulfonamides (sulfamethoxazole, sulfisoxasole, sulfadiazine) from theophylline has been achieved by minor procedural modifications [102-104,111]. Acetazolamide was reported [105] to coelute with theophylline using the Orcutt et al. procedure. Clark [106] reported that pH adjustment before extraction could provide a nearly complete separation of these two drugs.

During the analysis of an American Association of Clinical Chemists TDM Quality Control Program Vial, Marion et al. [107] found a potential interference of procainamide with their slight modification of the Orcutt et al. theophylline procedure. The interference occurred with a μBondapak C_{18} column dedicated to theophylline and acetaminophen serum analysis. The column had been used 7 months with approximately 900 injections. Their quality control program had indicated no problem with the assay system other than a slight increase in retention time with age. A new column gave complete baseline separation. The authors emphasize that long-term use of HPLC columns requires closer attention to possible interferences and the value of participating in external proficiency surveys.

A potential for interference exists in all of the liquid chromatographic procedures. It is necessary to minimize the incidence and to recognize potential problems quickly.

V. RELIABILITY OF ANALYTICAL PROCEDURES

In 1949, Schack and Waxler [113] developed the first analytical procedure based on ultraviolet spectroscopy for routine clinical evaluation of theophylline concentrations in biological fluids. The procedure requires relatively large volumes, and several commonly used drugs besides other xanthines interfere [114]. Gas-liquid chromatography provided specificity, but in most procedures, extraction-back extraction and derivatization are required [115-117]. If the therapeutic drug monitoring survey of the College of American Pathologists is an adequate reflection of practice in the clinical laboratories, essentially all laboratories had stopped using spectrophotometry or gas-liquid chromatography for their theophylline serum determinations by mid-1982. Their survey, based on 1000 laboratories, would indicate that about 10% each are using liquid chromatography and radioimmunoassay procedures [118]; 70% use on the the enzyme/spectrophotometric procedures [119-120]; and 5% each fluorescent immunoassay [121-122] or nephelometric inhibition immunoassay [123]. The latter, being new, is showing the most rapid growth in the number of laboratories trying the system. Another new theophylline assay procedure is by isotachophoresis [124].

The number of laboratories using liquid chromatography is stable. A discussion of these methodologies compared to LC constitutes Chap. 3 of this book. Several investigators have compared one analytical procedure to another with slightly varying results but with all showing high correlations [44,45,56,58,71,80,93,97,101,108-109,112,125-129]. When a procedure is being developed and under very strict quality control, coefficients of variation of 2-3% are reported. However, if the CAP drug monitoring survey is representative, then all of the various analytical procedures have operational coefficients of variation of about 10%. Considering the clinical application of the drug concentration determination, a coefficient of variation within 10% is adequate.

The individual laboratory's reliability is more important than the analytical procedure per se. Bonham et al. [130] studied the reliability of theophylline serum concentration determination using simulated patient samples in all of the clinical laboratories in one state. Eight months after the laboratories agreed to participate, three aliquots from a large pooled sample were submitted at 2- to 8-day intervals through local private physicians. The comparison control values were determined from the analysis of 65 aliquots over 4 months, giving a mean and S.E.M. of 15.7 ± 0.1 μg/ml with a coefficient of variation of 5.7% (Fig. 3). In this study, half of the laboratories reported at least one value, and 25% two or more values, outside the 95% confidence limits. There was no significant difference in performance between hospital laboratories and referral laboratories or between the methodologies of HPLC and EMIT. Finally, the reliability of the laboratory did not correlate with the fee charged.

Since standard theophylline assay procedures with established accuracy were used, the percentage of laboratories reporting erroneous results would indicate inconsistent performance. The authors conclude "that practitioners cannot rely on the results of a laboratory to adjust theophylline dosage unless the reliability of that laboratory has been established with blinded specimens disguised as patient samples, so that not only the method, but also the routine handling of samples and test performance are examined " [130].

VI. CONCLUSION

For the management of asthma and neonatal apnea, there is a well-documented need to monitor serum theophylline concentrations to maximize efficacy and minimize toxicity. Any one of the several versions of liquid chromatography as well as other analytical methodologies can give accurate determinations of theophylline concentration in serum. Because these measurements are used clinically to determine dosage, either poor laboratory performance or the occurrence of an unusual interference could result in an inappropriate clinical decision.

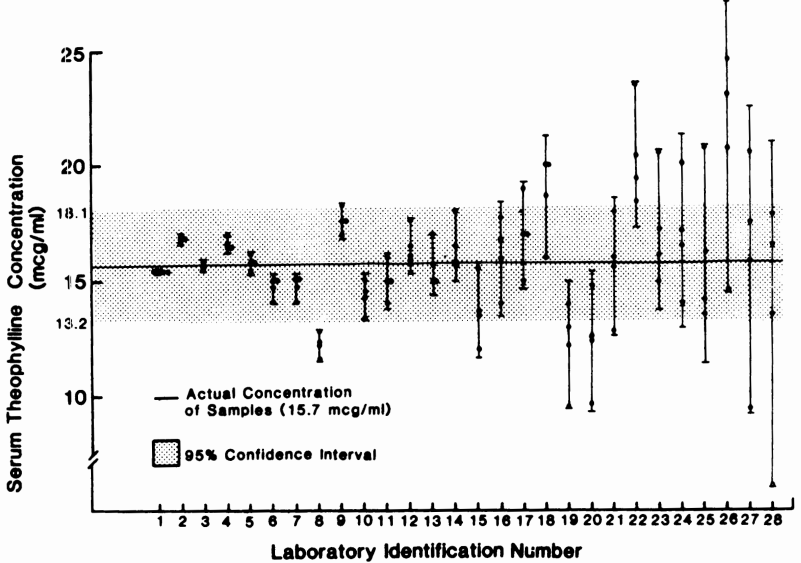

Figure 3 Reliability of serum theophylline concentration determinations in simulated patient samples. The 95% confidence interval is the defined range of acceptable concentrations. Solid circles represent mean values. Bars represent 2 S.E.M. above and below the mean. The open circles are the actual reported values (From Ref. 130).

There is no substitute for an effective quality control program and a vigilant technical staff in the operation of a drug monitoring laboratory.

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10

ANTIBIOTICS

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I. INTRODUCTION

Antimicrobial agents are very widely used in clinical practice. A recent study of 43 hospitals in England showed, for example, that of greater than 18,000 patients, nearly one quarter were receiving antimicrobials [1]. Over one billion dollars worth of antiinfectives were purchased in 1982 by hospitals alone. Antimicrobial agents clearly are important analytes, and they are well suited for determination by modern liquid chromatography (LC). The liquid chromatographic determination of antibiotics in biological samples is a somewhat diverse topic because of the disparate chemical structures of the many classes of clinically effective antibiotics. There have been several reviews of this subject [2-5], but these are either quite limited in scope or are now out of date. There has, however, been a recent review of the LC determination of antifungals [6]. An encyclopedic summary of recent references on LC for determination of antibiotics is also available [7].

This chapter attempts to cover in depth the LC determinations of the more important antibiotics and to extract some general principles. Those antibiotics reviewed here were chosen principally on the basis of their current (or future) importance to therapeutic medicine [8]. The literature reviewed includes up to 1982.

II. GENERAL CONSIDERATIONS

The rationale for therapeutic drug monitoring in general has been discussed in detail in Chap. 2 of this volume. There are several reasons

why it is desirable to determine the levels of an antibiotic in body fluids, usually serum. The reasons relate to safety and efficacy considerations: is there enough antimicrobial agent present at the site of infection to inhibit the infectious organism, but not enough to cause toxicity? For antimicrobial drugs with relatively low therapeutic indexes, such as aminoglycosides and chloramphenicol, it is very important to monitor their concentrations in blood. For antibiotics with higher therapeutic indexes, such as penicillins and cephalosporins, blood levels may be determined only in cases where a therapeutic failure has occurred, or where impaired renal function exists. The number of assays requested for the different antibiotics reflects these considerations. Aminoglycosides represent the majority of the requested determinations, greater than those for chloramphenicol and the other antimicrobials combined [5,9].

While considering the clinical laboratory use of LC for the determination of blood levels of antibiotics, it is important to note that the vast majority of such assays are currently performed by microbiological assay, radioimmunoassay, or radioenzymatic assay, rather than by LC. The characteristics of these types of methods are discussed in general in Chap. 3 of this volume. When the various assays are applied specifically to the determination of antibiotics, it is found that in most cases the LC assays are more precise and more accurate. The other assays generally have a higher sample capacity (i.e., the number of assays which can be "processed" simultaneously) and require less sample preparation. The immunoassays (and in some instances the microbiological assays) frequently have greater absolute sensitivity. The special advantage of LC is its specificity. LC assays are much less subject to interference than the competitive assays. This is particularly important considering that a patient may receive multiple antibiotics as part of a therapeutic regimen, or their records of treatments may be incomplete. For example, Reeves and Holt found that 19% of the sera submitted to their laboratory for assay contained undisclosed antibiotics [10]. In addition, LC can distinguish metabolites of antibiotics from the parent compound, as well as multiple isomers of the same compound where such isomerism exists. These last points are illustrated in the subsequent discussions of specific antibiotics.

The specific way in which any assay is developed depends upon the purpose for which it is intended. When the methods reported in the literature for determination of antibiotics in serum are considered, it is apparent that there is an evolution of methodology. The procedures developed during the clinical trial phase of a compound's life are oriented towards estimation of pharmacokinetic parameters. For this purpose the HPLC assay needs to be specific only in that it will resolve multiple forms and/or metabolites of the drug under study. The method need not resolve commonly administered drugs or other drugs of the same therapeutic type or category, since these will not be administered during controlled studies. These early methods need

to be accurate and precise over a large range of analyte concentrations, so that good pharmacokinetic data can be obtained. For routine therapeutic drug monitoring, the requirements are different. The method should be fast and specific for the drug in the presence of other drugs. If possible, the method should be generally applicable, so that multiple compounds may be determined (either individually or in a mixture) by the same procedure. Accuracy and precision are desired but are not as critical as during pharmacokinetic studies. A good method may be suitable for either pharmacokinetics or therapeutic drug monitoring, and a well-developed method will be readily adaptable from one purpose to the other.

The preparation of samples for LC usually involves protein precipitation, liquid–liquid or liquid–solid extractions. With regard specifically to antibiotics, there is one aspect of sample preparation which merits additional comments. That aspect is the deleterious effects which injected sample solvents can have on chromatographic performance. It has long been recognized by chromatographers that there are certain limitations as to the solvent in which an analyte should be dissolved for LC. These limitations encompass both the nature of the solvent and the amount injected. However, even a brief review of the chromatographic literature in general shows that many practicing chromatographers are insufficiently aware of these phenomena. This is particularly true for LC determinations of antibiotics in body fluids. Injected sample solvent problems are a weakness of many of the publications discussed later in this chapter.

Injected sample solvent problems can occur whenever the sample solvent which is injected is a stronger eluent than the mobile phase, as shown by Fig. 1. Moxam is a β-lactam antibiotic consisting of two diastereomers. These can be seen to be well resolved in the dashed upper trace. The moxam was dissolved in the mobile phase (which consisted of 0.1 M ammonium acetate and 5% acetonitrile) and injected. The injection volume was 50 μl. Injection of the same amount of moxam dissolved in 60% acetonitrile/40% water resulted in the lower trace. In this case, 60% CH_3CN is a much stronger eluent than the mobile phase. As a result, most of the moxam eluted with the large, injected acetonitrile peak (retention time, about 3 min). The moxam peaks remaining at their expected elution time clearly are broader, resulting in significant loss in sensitivity. Additional problems include band distortion, changes in retention time, and poor precision.

The case discussed above was one in which the volume fraction of the organic solvent was too high. Other aspects of injected solvents which can cause problems are a difference in pH between the sample solvent and the mobile phase, a difference in ionic strength, the lack of an ion-pairing reagent or chelating agents in the sample solvent which are present in the mobile phase, and vice versa. Frequently these problems arise inadvertantly from required extraction or serum protein precipitation steps, as shown in Fig. 2. A trichloroacetic acid precipitation of plasma proteins was carried out on plasma spiked with

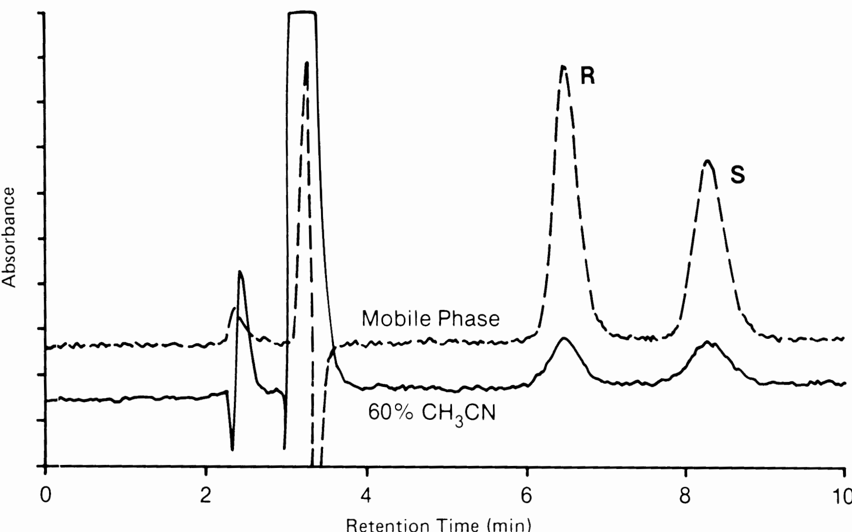

Figure 1 Chromatograms of 0.5 μg of moxalactam injected in two different solvents. The peaks labeled R and S are the two diastereoisomers. Conditions: pH 6.5, 0.1 M ammonium acetate/acetonitrile (95:5); flow-rate, 1.5 ml/min; injection volume, 50 μl; 30 cm × 4.6 mm Chromegabond C_{18} column; 270 nm detection.

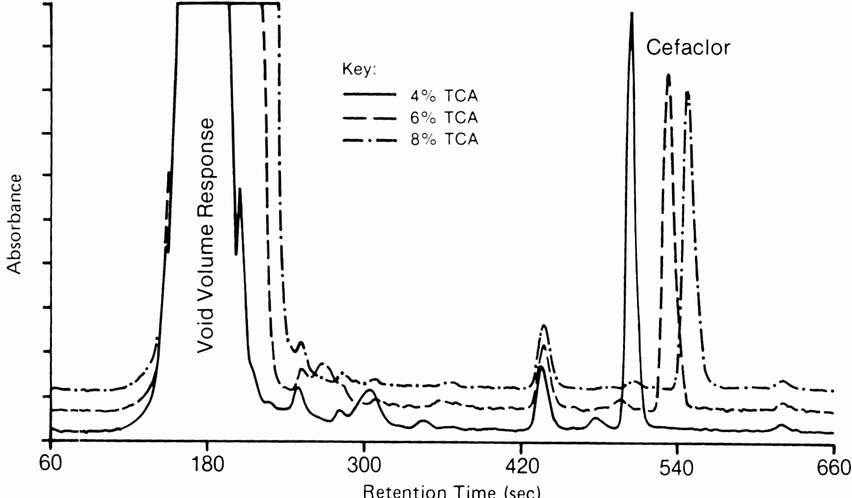

Figure 2 Chromatograms of a cefaclor-containing plasma. Sample treated with 1 volume of trichloroacetic acid. Conditions: pH 2.75, 0.2 M acetate/CH_3CN (10:3); flow-rate, 1 ml/min; injection volume, 50 μl; 25 cm RP-8 (Merck) column; 262-nm detection.

cefaclor. Depending upon the concentration of TCA used, different retention times were obtained for cefaclor. A large difference in peak heights resulted for these samples, in spite of identical added amounts of cefaclor.

Two possible solutions to injected solvent-derived problems are to keep the injection volume as low as practical and to make the sample solvent as similar to the mobile phase as possible. Where a sample solvent must be greatly different in composition from the mobile phase, it should be as "weak" an eluent as possible. This topic is discussed in detail elsewhere [11], but it is prudent to be wary of injected solvent problems when considering determining an antibiotic by LC.

III. REVIEW OF LIQUID CHROMATOGRAPHIC METHODS

A. Aminoglycosides

1. Pharmacology

The aminoglycosides are a potent group of antibiotics containing amino sugars in glycosidic linkage. Streptomycin, initially isolated in 1943, was the first of this group to be introduced. A series of related compounds have followed. The structures of the most important of the aminoglycosides currently available are shown in Fig. 3. Streptomycin and neomycin have been omitted from this discussion because requests for their determination are infrequent [5,9]. In addition, no LC determinations of these two aminoglycosides in biological fluids have been reported. Requests for kanamycin plasma levels are even more infrequent; thus kanamycin has been omitted also.

The mechanism of action of the aminoglycosides involves active transport across the bacterial cell membrane, followed probably by direct inhibition of protein synthesis at the ribosome. The details of the mechanism differ with each drug. The antibacterial spectrum of these aminoglycosides primarily encompasses aerobic, gram-negative bacilli.

The aminoglycosides are polycations and thus are poorly absorbed after oral administration. All may be administered intraveneously or intramuscularly. After intramuscular doses they are rapidly absorbed. Aminoglycosides are not metabolized or bound to plasma proteins and are excreted almost entirely by the kidney.

Desirable blood levels for these aminoglycosides are on the order of 1-10 μg/ml, with the exception of amikacin, for which higher levels are required. Their serum half-lives are all approximately 2 hr. All of the aminoglycosides can cause ototoxicity and nephrotoxicity. Although the precise relationship of blood levels to the development of toxicity is not known, the therapeutic index for aminoglycosides is somewhat low. As a result there is a particular need for close monitoring of the concentrations of aminoglycosides in serum. The majority

Gentamicin Sisomicin Netilmicin

Tobramycin Amikacin

Figure 3 Structures of aminoglycosides.

of requests for the determinations of antibiotic levels in clinical laboratories involve aminoglycosides. These assays normally are performed by microbiological, radioenzymatic, radioimmunological methods, or competitive binding assays including EMIT, FPIA and others [12].

2. LC Methodology

A number of LC methods have been reported for amikacin [13-15], netilmicin [22,24,28], sisomicin [23], and tobramycin [13,22,25-29]. Gentamicin has been the most frequently studied of the group [13, 16-23,28,30]. The relatively large number of reports of methods for determining gentamicin and the other aminoglycosides in serum or plasma probably reflects two factors: the importance of determining these compounds and the lack of completely satisfactory LC methods for their determination.

The lack of completely satisfactory methods for determination of aminoglycosides in serum is due to the difficulty of working with them. They do not have any chromophores and hence must be derivatized in order to be detected at the μg/ml level in serum. The experimental approaches are summarized in Table 1.

Table 1 Approaches to LC Determinations of Aminoglycosides

Procedural sequence	Analytes	References
1. Sephadex extraction/ion-pair reversed-phase LC/OPA	Gentamicin Amikacin, tobramycin	13
2. Precipitate proteins/ion-pair reversed-phase LC/OPA	Gentamicin	20
3. Silica extraction/OPA/reversed-phase LC	Amikacin Gentamicin Tobramycin	14,15,17, 19,26
4. Amberlite extraction/OPA/reversed-phase LC	Gentamicin Netilmicin Tobramycin	28
5. Solvent extraction/OPA/reversed-phase LC	Gentamicin Netilmicin Tobramycin	22,25,29
6. Solvent extraction/dansylation/reversed-phase LC	Gentamicin Netilmicin	16,24
7. Solvent extraction/fluorescamine/ion-exchange LC	Gentamicin	18
8. Solvent extraction/benzenesulfonyl chloride/normal-phase LC	Gentamicin	21
9. Solvent extraction/fluorodinitrobenzene/reversed-phase LC	Gentamicin Sisomicin Tobramycin	23,27
10. Precipitate proteins/trinitrobenzenesulfonic acid/reversed-phase LC	Tobramycin	29

All but four of the papers reported derivatizations with fluorescent reagents (approaches 1-7), principally *o*-phthalaldehyde (OPA). UV-absorbing derivatives were used in approaches 8, 9, and 10. The use of derivatization reagents in a procedure can add additional problems. A second source of difficulty in working with aminoglycosides is their polarity. Aminoglycosides are sufficiently polar that to extract them with water-immiscible organic solvents is impossible (a common approach to sample cleanup). Also, they are poorly retained by conventional reversed-phase chromatography. As a result of these difficulties, LC

determinations of aminoglycosides are relatively complex and labor intensive, requiring relatively skilled personnel.

A number of specific comments about the approaches in Table 1 are in order. First, gentamicin determinations were attempted by all but one of the reported approaches. Second, only the first two procedures listed in Table 1 use postcolumn derivatization. In order to retain the polar aminoglycosides on reversed-phase LC (without first derivatizing them with a hydrophobic derivatization reagent), an ion-pairing reagent was added to the mobile phase. The separations obtained by Anhalt and Brown using this approach are still probably as good as any reported (see Figs. 4 and 5).

The most frequent approach to these determinations has involved column extraction of the aminoglycoside, usually on silica, followed by OPA derivatization, elution from the silica column, and chromatography. However, one investigator has reported difficulty in reproducing recoveries from the silica column, particularly with tobramycin. The use of an amberlite column in place of the silica was suggested [28]. Also, several papers have reported substituting solvent extractions for column cleanups. Unfortunately for all of these methods, the OPA derivatives are not very stable, necessitating rapid injection of derivatized extracts and making unattended automatic injections of extracts impossible. No particular advantage relative to OPA was demonstrated for the dansyl and fluorescamine derivatives (approaches 6 and 7). Formation of a UV-absorbing derivative allows the use of conventional UV detectors, which are more widely available. For the benzenesulfonyl chloride and fluorodinitrobenzene derivatives, the reaction conditions are rather strenuous, and yields are low or extractions complex. Derivatization with 2,4,6-trinitrobenzenesulfonic acid appears more promising [29], although the absolute recovery of this approach was not established. Many of the reported procedures are potentially subject to injected solvent-derived problems. For example, the direct injection of ethyl acetate containing the dansyl derivative of gentamicin caused nonlinearity when more than 2 μl was injected into the LC! [16].

Despite the inherent difficulties, workable LC methods are available for determining each of the aminoglycosides. The precisions reported for the methods in Table 1 were good. Detection limits were all between 0.2 and 1 μg/ml, which is quite sufficient for therapeutic drug monitoring. No chromatographic interferences were noted for any of the assays, other than the interference of one aminoglycoside in the determination of another.

The procedures which involve column chromatography as part of their sample cleanup can now potentially be partially automated. Two instrument systems for automation of column chromatography, the DuPont Prep 1 and Analytichem's AACS, have recently become available. The DuPont unit uses centrifugal force to pass a sample, a wash solvent, and an eluting solvent over a short column. Up to 12 samples

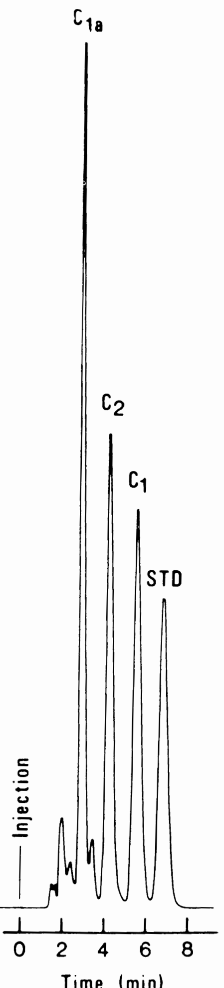

Figure 4 Representative chromatogram of an extract of serum which contained gentamicin (10 μg/ml) and 1-*N*-acetylgentamicin (STD). For conditions, see Ref. [13]. (Reprinted with permission from *Clinical Chemistry*.)

may be processed simultaneously. After collection of eluted species, the unit will evaporate the elution solvent. The sample must then be manually reconstituted and injected, or loaded onto an autoinjector. Columns containing a variety of packing materials are available. The AACS utilizes racks of small columns. The samples are loaded onto the columns off-line. The rack is then placed into the unit, which

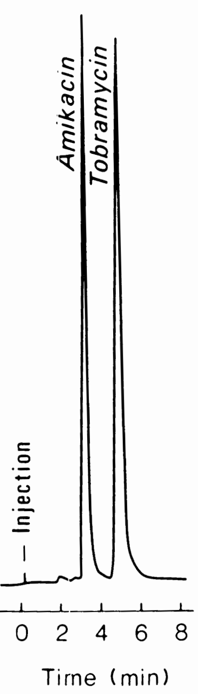

Figure 5 Chromatogram of an extract of serum which contained amikacin (5 μg/ml) and tobramycin (5 μg/ml). For conditions, see Ref. [13]. (Reprinted with permission from *Clinical Chemistry.*)

provides for sequential connection of the individual columns in the rack to the analytical column. Thus direct automated injection is accomplished. The timing of the connection of the small columns to the analytical column can be controlled so that interferences need not be transferred onto the analytical column. This can provide some additional selectivity. Another piece of equipment which could be applied to the automation of aminglycoside sample preparation is the Micromeretics Model 725 automatic injector. This injector can be equipped to mix the contents of two vials at the time of injection. With one vial containing the sample, and the other OPA, derivatization could conveniently be accomplished immediately (and presumably reproducibly) prior to injection.

The most promising approach to determination of aminoglycosides may be that of Kubo an co-workers [20]. In their procedure for determining gentamicin, the proteins in 20-μl samples of serum were precipitated by addition of 100 μl of methanol. After centrifugation, 200 μl of an aqueous solution of the ion-pairing reagent used for

chromatography was added. This solution was centrifuged again and a 240-μl aliquot of the supernatant injected onto the LC. After ion-pairing reversed-phase LC, the components were derivatized with OPA and detected by their fluorescence. The small sample required for this method and straightforward sample preparation make this approach attractive. The required postcolumn derivatization can now be conveniently accomplished with one of the self-contained units for derivatization which have recently become available from several manufacturers.

3. *Conclusions*

The advantages and disadvantages of LC determination of gentamicin relative to a bioassay, a radioenzymatic assay, and a homogeneous enzyme immunoassay were well summarized by Delaney et al. [17]. Assay results by all four methodologies correlated well with each other. The LC determination exhibited better precision than the microbiological and radioenzymatic assays and was the most accurate of the four assays. LC also has the advantage of providing greater specificity. This is particularly evident, of course, with gentamicin, where three or more components may be resolved and individually determined (see, for example Fig. 4). As noted above, the disadvantages of LC gentamicin determinations were the equipment, the time-consuming sample preparations, and the skilled personnel required. These conclusions certainly apply as well to the LC determinations of the other aminoglycosides.

Although LC is currently not widely used in the clinical laboratory for determination of aminoglycosides, it could be, if a convenient and general procedure were available. The prospects for more convenient sample preparation procedures were discussed above. The barrier to a general LC procedure is the need to chromatographically resolve all of the aminoglycosides. This may be achieved some day although to date only one or two have been assayed together.

B. Penicillins

1. *β-Lactams*

Over half of the antibiotics in use today contain a β-lactam ring. These agents are principally classified either as penicillins or cephalosporins. Penicillins consist of the β-lactam ring, a fused thiazolidine ring and various acyl-containing substituents at the 6-amino position (Table 2). Cephalosporins have a dihydrothiazine ring fused to the β-lactam ring and various substituents at the 3- and 7-amino positions (Table 3). The structures of several additional β-lactams are depicted in Table 4. The mechanism of action of β-lactams involves inhibition of the cross-linking of peptidoglycans, which normally provide mechanical stability to the bacterial cell walls. Recently, x-ray crystallography of such an

Table 2 Penicillins

R–C(=O)–NH– [penicillin nucleus: S, N, CH_3, CH_3, COOH, O]

Name	Side chain (R)	Activity class[a]	References
Benzylpenicillin (Penicillin G)	C_6H_5–CH_2—	I	None
Phenoxymethylpenicillin (Pencillin V)	C_6H_5–OCH_2—	I	None
Oxacillin (R_1=R_2=H)	[2-(R_1,R_2-phenyl)-5-CH_3-isoxazol-4-yl; N, O]	II	32,33
Cloxacillin (R_1=Cl, R_2=H)		II	32-35
Dicloxacillin (R_1=R_2=Cl)		II	32,33
Nafcillin	[naphthyl, O_2CH_5]	II	33,35
Ampicillin (R=H)	R–C_6H_4–CH(NH_2)—	III	36,37
Amoxicillin (R=OH)		III	36,38,85

Carbenicillin	CH— COOH	IV	None
Ticarcillin	CH— S O O CH_3	IV	45
Azlocillin (R=H) Mezlocillin ($R=SO_2CH_3$)	CH— NHCO N O N R	V VI	39 39
Piperacillin	CH— HNCO N O N H O	V	40-42
Mecillinam	N—CH = b	VII	37,43,44

[a]I = benzylpenicillin-like activity, II = penicillinase-resistant, III = extended spectrum penicillins, IV = activity against *P. aeruginosa*, V = activity against *Pseudomonas*, VI = activity against *Klebsiella*, VII = activity against *Enterobacteriaceae*.
[b]Double bonded directly to 6-amino group.

enzyme, a penicillin-sensitive D-alanyl-carboxypeptidase-transpeptidase, has provided direct evidence of the interaction of β-lactams with these enzymes [31]. As cell growth continues without sufficient support, cell lysis can follow.

2. Pharmacology

Originally isolated aroung 1940, penicillins remain the drugs of choice for a large number of infections. The penicillins in Table 2 are grouped according to activity. Not included here are prodrugs such as hetacillin, bacmecillinam, and pivampicillin, which are rapidly converted during absorption to compounds listed in Table 2. The prodrugs themselves are not easily detectable in plasma. Penicillins may be administered orally or by intramuscular or intraveneous injection, depending upon the compound. Their duration of action is relatively short, with serum half-lives ranging from 25 to 80 min. Penicillins are primarily excreted in the urine. Metabolic inactivation results primarily from penicillinase hydrolysis of the β-lactam ring to give the inactive penicilloic acid derivative.

Plasma levels of clinical significance for the older penicillins are generally in the range of 1-25 μg/ml. For the anti-pseudomonal and ureido penicillins, plasma levels can be as high as 400 μg/ml. Penicillins may cause dose-related neurotoxicity when high levels are present in the cerebrospinal fluid. However, there is a large margin of safety between therapeutic and toxic levels. Plasma levels are most frequently determined by microbiological assays.

3. LC Methodology for Selected Penicillins

Presumably because penicillins as a class are relatively safe and many were established therapeutic agents before the advent of modern LC, only 20 methods for the LC determination of various of these 14 penicillins in biological fluids have been reported. Among these are five which were developed for urine assays only, and thus are not discussed here. It appears that no methods have been reported for penicillin G, penicillin V, and carbenicillin. Methods for the other penicillins are referenced in Table 2.

Two approaches to preparation of penicillin samples for LC have been taken; namely, simple precipitation and extraction procedures. Trichloroacetic acid [37], perchloric acid [32,36], dimethylformamide [41], and acetonitrile [33,34,44] have been used as precipitation reagents. Although simple and rapid, the use of acids may cause degradation of some penicillins [34]. Precipitation with acetonitrile or dimethylformamide gives a sample "extract" for which injected solvent effects may be observed, so that injection volumes should be kept low (see Sec. II above). Solvent extractions have generally been performed with solvents like chloroform, due to the relative polarity of penicillins.

These procedures afford some cleanup and concentration of sample extracts. Liquid-solid extraction of two penicillins has also been reported [39].

A wide variety of chromatographic conditions have been used in penicillin determinations. One aspect of the chromatography of penicillins which has been virtually unnoticed by those doing therapeutic drug monitoring is that nearly half of the compounds in Table 2 contain an asymmetric carbon in the side chain. Since the β-lactam and the thiazolidine rings contain asymmetric centers, two side-chain diastereomers are possible. In many cases the isomers have different microbiological activities. LC generally is an excellent tool for isomeric separations (see, for example, Fig. 1), and its successful application to isomeric separations of a number of penicillins has recently been reported by Hoogmartens et al. [46]. Although they did not consider plasma samples, they demonstrated ready separation of eight pairs of isomers. Such separations can be extended to plasma assays; yet only one such separation has been reported for penicillins [45], and it was neither recognized nor utilized by the investigators.

A single phenyl ring is responsible for the ultraviolet absorption of all but two of the penicillins, so that they are commonly detected in the region of 210-240 nm using variable-wavelength detectors. Detection limits are generally on the order of 0.5-1 μg/ml of plasma. Detection of amoxicillin and ampicillin can be enhanced by derivatization with fluorescamine [47]. Amoxicillin may also be detected with enhanced selectivity and sensitivity by liquid chromatography with electrochemical detection [48]. Westerlund and co-workers reported an interesting approach which gives enhanced absorbance at 310 nm, where endogeneous interferences are much less detectable [37]. Derivatives were formed via addition of imidazole, which attacks the β-lactam bond, and mercuric chloride, which reacts with the sulfur of the fused ring. The reaction is applicable to cephalosporins as well as penicillins, and does not detect inactive derivatives lacking the intact β-lactam ring. Unfortunately, the above advantages are probably outweighed by the lengthy reaction time (5-20 min at 40°C) needed for derivatization, which necessitates the use of air-segmented postcolumn derivatization equipment. Precolumn derivatization is probably precluded by the instability of the derivatives.

The papers of Thijssen [32] and of Rudrick and Bawdon [33] merit special mention because they describe procedures for determining multiple penicillins, rather than just one or two. Both papers dealt with the isoxazolyl penicillins (oxacillin and related compounds). One of the separations obtained is depicted in Fig. 6. Their procedures were thoroughly investigated and well tested. Such procedures, developed to include even more compounds, could find great utility in the clinical lab.

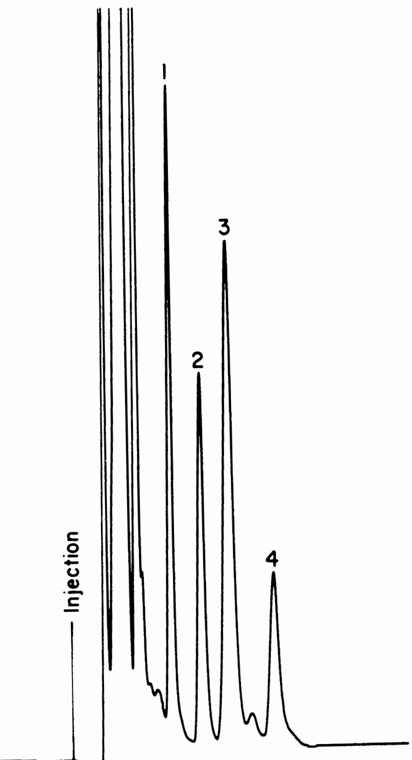

Figure 6 Chromatogram of an extract of serum which contained 20 μg/ml of each of the following: (1) oxacillin, (2) cloxacillin, (3) nafcillin, and (4) dicloxacillin. For conditions, see Ref. 32.

C. Cephalosporins

1. *Pharmacology*

The first source of cephalosporins was an organism isolated from Sardinian seawater in 1948. When the active nucleus was isolated and semisynthetic derivatives prepared by addition of side chains, a new, highly active class of β-lactams was realized. The structures of a number of those currently available are shown in Table 3. Some closely related β-lactams are depicted in Table 4. The cephalosporins are active against a variety of bacteria, including both gram-positive and gram-negative species. There is considerable variation among them in regard to activity against any particular organism. They also differ as to routes of administration and distribution. Several are active orally, but most require I.M. or I.V. injection to achieve thera-

Table 3 Cephalosporins

(General structure: R_1–C(=O)–HN– cephem nucleus with S, N, =O, R_2, COOH)

Name	R_1	R_2	References
Cefaclor	NH_2	Cl	49
Cefroxadine	NH_2	$—OCH_3$	50,51
Cephalexin	NH_2	$—CH_3$	51-53,80
Cefamandole	OH	$—CH_2—S—$ (N—N, N—N, CH_3)	54-57
Cefoperazone	HO—, NH, O=, N, O, N, O	"	55,58-60
Cefmenoxime	H_2N—, S, N, N, OCH_3	"	61-63
Cefotiam	H_2N—, S, N	$—CH_2—S—$ (N, N, N, N, N)	51,62,83

Table 3 (Continued)

Name	R_1	R_2	References
Cefotaxime	H_2N, S, N, N, OCH_3	$-CH_2-O-$, O, CH_3	51,54,55, 64-66
Ceftizoxime	″	H	67,83
Ceftriaxone	″	$-CH_2-S-$, N, O, O, N–N, H, CH_3	68
Ceftazidime	H_2N, S, N, N, O, COOH	$-CH_2-\overset{+}{N}$	69
Cefsulodin	[structure], SO_3Na	$-CH_2-\overset{+}{N}$, $CONH_2$	51,62,70
Cefazolin	N=, N, N=N	$-CH_2-S-$, N–N, S, CH_3	54,55
Cefuroxime	O, N, OCH_3	$-CH_2-OCONH_2$	51,55
Cephalothin	S	$-CH_2-OCOCH_3$	55,71

Table 4 Other Beta-Lactams

Name	Structure	References
Cefmetazole		72
Cefoxitin		54,55,58, 66,73,74
Moxalactam		75-79
Clavulanic acid		85
Aztreonam		86,87

peutic plasma levels. Protein binding in plasma varies from 20 to 90%. Plasma half-lives for cephalosporins generally fall in the range of 1-3 hr. All cephalosporins are excreted by the kidney, and some are subject to metabolic deactivation either bacterially or by the host. Two exceptions are cefoperazone and ceftriaxone, which are principally excreted via the liver and into the bile.

Plasma levels of clinical significance are generally in the range from 1 to 200 μg per ml. Dosage adjustments may be indicated for renally impaired individuals, but side effects due to cephalosporins are quite

infrequent. Plasma levels are most frequently determined by microbiological assay.

2. LC Methodology for Selected Cephalosporins

Like the penicillins, the cephalosporins have not received attention from clinical chromatographers in proportion to their number and widespread use. This is undoubtedly due to their relative safety, so that plasma level determinations are infrequently requested. Reported methods are referenced in Tables 3 and 4.

A variety of sample preparation techniques for plasma level determinations of various cephalosporins have been reported, but two-thirds of the reported methods used simple precipitation procedures. Due to the instability of many cephalosporins in acidic solutions, the precipitation of plasma proteins has been most commonly accomplished by the addition of from 1 to 5 volumes of a solvent such as a methanol, rather than by addition of strong acids (e.g., trichloroacetic acid). It may be of advantage to add a small amount of acid, in addition to the organic solvent, to lower the pH of the supernatant below 7, enhancing the stability of the cephalosporin [75]. Adding acid in a second step has the added effect of reducing the percent organic solvent in the injected solution. Since cephalosporins require relatively low amounts of methanol or acetonitrile to elute them from reversed-phase columns (5-30%), the injection of 50- to 100-μl volumes of supernatants which contain 50-84% organic solvent can lead to serious band broadening, with a concomittant loss of resolution. Peak heights are immediately affected (see Fig. 1), and in many cases even peak area measurements become inaccurate. Injected solvent problems can also be minimized (and sensitivity enhanced) by using only 1 volume of organic solvent to precipitate proteins. This requires the use of a high-speed centrifuge (>10,000 *g*) to obtain a good precipitate. Although the supernatant which results is not as clean as can be obtained with 4 or 5 volumes of solvent, inclusion of a precolumn protects the main column, and no other adverse effects are observed. Injected-solvent effects on cephalosporins have been noted by several authors.

Several plasma preparation procedures other than simple precipitation have been reported for cephalosporins, including solvent extraction, anion-exchange chromatography, and ultrafiltration. All of these approaches should yield cleaner extracts. Solvent extractions have been infrequently reported, due to the relative hydrophilicity of most cephalosporins. Anion-exchange procedures [66,67] unfortunately result in greater dilution of the samples than that seen for simple precipitation procedures. The application of ultrafiltration should provide the advantage of allowing the separate determination of free (as opposed to plasma-protein bound) levels of antibiotics [63,70]. Total levels are normally determined, such as in the precipitation procedures described above.

All of the reported methods for cephalosporins have employed ultraviolet detection, the majority operating at 254 nm. This is a reasonable choice since the absorption maximum due to the cephalosporin nucleus itself is around 266 nm. Depending upon the contribution of the two side chains to the absorbance spectrum, as well as the characteristics of any plasma interferences, wavelengths slightly higher or lower than 254 nm have been found to be optimum. If extreme sensitivity were necessary, it might be possible to substitute postcolumn derivatization and longer wavelength or fluorescence detection [37,81].

The detection limits reported almost all fall in the range of 0.1-1.0 μg/ml, which is reasonable, given the therapeutic ranges of cephalosporins. The accuracy of HPLC determination of cephalosporins has frequently been verified by comparison against a microbiological assay. Precision has normally been estimated by assaying a sample several times during a single day. Thorough multiple-day studies of precision are unusual, and many reported methods were not characterized in any way. Nonetheless, HPLC determinations of cephalosporins can, in general, be expected to have an overall precision better than 10%.

Two additional characteristics of cephalosporin assays that have been checked by only a few authors are assay interferences and the stability of samples. Since the conventional reversed-phase chromatography used for cephalosporins requires only low percentages of organic modifiers in the mobile phase, and since sample preparation procedures are simple and nonselective, it may be expected that a number of drugs commonly encountered in a clinical setting might interfere with the chromatography of any given cephalosporin. The only papers reporting having looked seriously at potential interferences observed minimal problems [54,58,66,71]. Further work here is merited, however. Similarly, only a few authors have carefully evaluated the stability of samples under various storage conditions, the stability of sample extracts, and the *absolute* recovery of their procedures. It is important to know what is required in terms of handling of a sample in order to maintain its integrity, and this is particularly true for cephalosporins, many of which are not optimally stable. It is also of value to know rates of degradation so that plasma levels may be back-calculated for samples which were improperly handled.

As was noted earlier with penicillins, a major limitation of reported HPLC determinations has been the lack of methods applicable to multiple cephalosporins. Until recently, only a maximum of two had been assayed simultaneously. Several groups have now addressed this limitation. Danzer reported a method for determining five cephalosporins together with chloramphenicol and an internal standard [54]. His procedure was a simple precipitation, the precision was good, and interferences were few. Unfortunately, the LC column used was a specialized one, not available in many laboratories. Khalil and co-workers have also made a preliminary report of a method for determining five cephalosporins [82].

A third group has systematically examined the solvent parameters necessary for simultaneous separation of 10 cephalosporins [84]. A low-pH mobile phase was chosen to maximize the retention of the cephalosporins relative to plasma interferences, and the mobile phase was buffered because of the sensitivity of t_R to changes in pH at low pH for some cephalosporins [83]. Systematic examination of combinations of methanol, acetonitrile, and tetrahydrofuran showed methanol to be the solvent of choice. (The future impact of automated examinations such as this one is discussed further in Sec. IV). Coupling the optimized chromatography to a simple precipitation procedure, the chromatogram depicted in Fig. 7 was obtained. The separation of eight cephalosporins is shown there, but the number could be even larger. Cephalothin and cefamandole eluted with retention times longer than 900 sec, and there clearly may be additional cephalosporins or penicillins resolved, even within the 15-min chromatogram depicted. This approach may be adapted to a truly general and widely applicable method for a class of antibiotics.

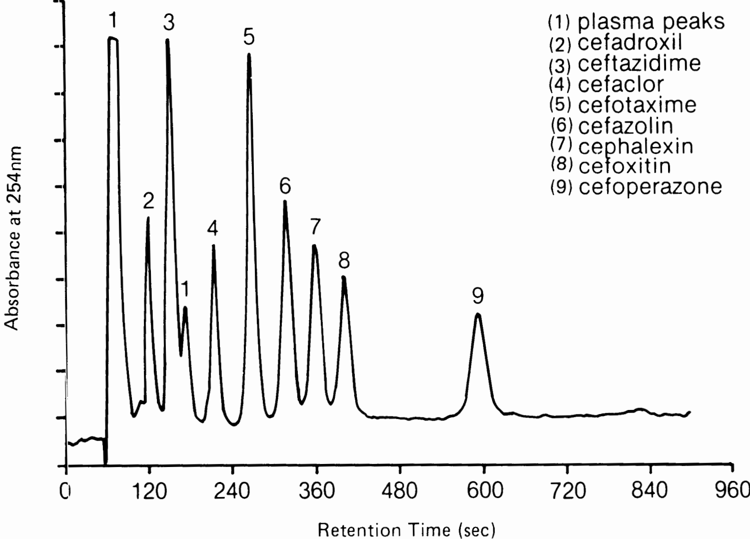

Figure 7 Chromatogram of an extract of plasma which contained 10 μg/ml of several cephalosporins. (1) plasma peaks, (2) cefadroxil, (3) ceftazidime, (4) cefaclor, (5) cefotaxime, (6) cefazolin, (7) cephalexin, (8) cefoxitin, (9) cefoperazone. Conditions: pH 3, 0.05 M lithium citrate/23% CH_3OH; flow-rate, 2 ml/min; injection vol., 40 μl; 15 cm Zorbax C_8 column at 35°C; 254 nm detection.

D. Chloramphenicol

1. Pharmacology

Chloramphenicol was first isolated from a soil sample collected in Venezuela in 1947. It became commercially available shortly thereafter. The structure of chloramphenicol is depicted in Fig. 8. Chloramphenicol has a relatively broad spectrum of antimicrobial activity. It acts by binding to the 50S ribosomal subunit in bacterial cells, thereby inhibiting protein synthesis. It has long been recognized that chloramphenicol can cause serious and fatal blood dyscrasias, principally aplastic anemia. A resurgence in the use of the drug has been caused, however, by the emergence of ampicillin-resistant *H. influenzae* strains. There are several other indications for chloramphenicol as well.

Chloramphenicol may be given orally or intravenously. It is rapidly absorbed after oral administration, with blood levels peaking in 1-2 hr. When given intraveneously, the sodium salt of the 3-monosuccinate ester is used as a prodrug. This derivative is inactive but is rapidly hydrolyzed in vivo. Chloramphenicol is widely distributed in body fluids. It is metabolized primarily to glucuronide esters, but other metabolites are also formed. In 24 hr, 75-90% of an oral dose is excreted in the urine, mostly as metabolites of chloramphenicaol. The half-life of the drug is on the order of 2.5 hr.

Desirable blood levels for chloramphenicol are in the range of 10-20 μg/ml in plasma. Concentrations greater than 25 μg/ml and prolonged courses of therapy are associated with reversible hematological toxicity. Blood level monitoring of neonates and adults with liver disease is particularly important. Chloramphenicol is most frequently assayed for by HPLC or microbiological assay.

2. LC Methodology

Chloramphenicol is the antibiotic for which LC is most frequently used in therapeutic drug monitoring. In contrast to the aminoglycosides, the LC determination of chloramphenicol in plasma is relatively straightforward. There have been at least 20 procedures reported for making this determination. They are conveniently differentiated on

Figure 8 Structure of chloramphenicol, R=R' =H; chloramphenicol-3-monosuccinate, R=H, R'=$COCH_2CH_2CO_2H$; chloroamphenicol-1-monosuccinate, R=$COCH_2CH_2CO_2H$, R=H.

the basis of just two factors: whether they involve extraction of the drug into an organic solvent or not, and whether they are capable of determining the monosuccinate esters of chloramphenicol in addition to chloramphenicol itself. Such a categorization is given in Table 5.

The relative merits of the precipitation and extraction approaches to determination of chloramphenicol are similar to those noted for many small organic analytes. The precipitation procedures are faster since they require minimal sample manipulations. As a result, they are also more reproducible, with RSDs under 5%. The RSDs reported for extraction procedures are all between 5 and 10%. Only a single extraction is required so that these procedures are relatively straightforward also. Theoretically the extraction procedures could give better detection limits, since the sample extract is concentrated during removal of the organic solvent, whereas the samples are diluted during precipitation procedures. Extraction is not as advantageous with chloramphenicol as it might be for some other analytes, since the nitro substituent of chloramphenicol is quite polar. Relatively polar solvents such as ethyl acetate and chloroform/isopropanol must therefore be used, and these offer less selectivity. Thus in practice, detection limits for the two approaches are comparable, all being on the order of 1 μg/ml. The extraction procedures do have the advantage of being less subject to interferences from other drugs. Koup and co-workers noted interferences when using a simple precipitation approach [92], and Petersdorf et al. used dual-wavelength monitoring to check the integrity of the chloramphenicol peak and noted evidence of interference in 2 of 15 samples [89]. Thus the choice of approach perhaps depends on the certainty with which the other constituents of a sample are known.

The second major division among the procedures revolves around the succinate esters of chloramphenicol, one of which (3-monosuccinate) is used for intravenous dosing. The other ester exists in equilibrium with the first at pH values near neutrality [101]. Since the succinates are inactive, the interest in them is strictly for pharmacokinetic evaluations. In order to maintain the integrity of the sample (i.e., prevent

Table 5 LC Determination of Chloramphenicol

Category	Procedure	Succinate esters determined?	References
A	Protein precipitation only	No	54,88-90
B	Solvent extraction	No	91-94
C	Protein precipitation only	Yes	95-97
D	Solvent extraction	Yes	98-100

interconversion of the esters), it is necessary to acidify the plasma promptly [96]. The major difference among procedures A + B in Table 5 and procedures C + D relates to the chromatography alone. The relative retention of the esters is sensitive to the mobile phase pH, due to the presence of the acid functionality. Retention of chloramphenicol is not sensitive to the pH. As a result, at low pH the esters elute after chloramphenicol and can be overly strongly retained [54]. At high pH the succinates elute before chloramphenicol and may be hidden under large coeluting plasma constituents which are not retained or are poorly retained on the column. At intermediate pH it is possible that the esters could interfere with quantitation of chloramphenicol itself. For the separation of these species, the chromatographic conditions of Wargin and co-workers [96] appear to be the best reported to date (see Fig. 9).

The characteristics of most of the procedures reported for chloramphenicol are quite good. Ultraviolet detection of the eluted chloramphenicol is quite convenient, since λ_{max} is at 278 nm, which is conveniently close to the 280-nm line available on most fixed-wavelength UV detectors. UV detection at 254 nm will suffice also. As noted above, the typical 1 μg/ml detection limits of these LC determinations are quite adequate for therapeutic drug monitoring. Additional sensitivity may be obtained through the use of electrochemical detection [102,103]. However, the electrochemical reaction involved is a reduction of the nitro group and detection in the reduction mode is much less convenient than for oxidations. With only one exception, the procedures in Table 5 require less than or equal to 100 μl of plasma, making them well suited for use with samples from neonates. The precision of all of the procedures is also acceptable for therapeutic drug monitoring. A number of possible internal standards have been utilized, most of which are commercially available. Thiamphenicol [93, 100] and the *N*-acetyl analogue of chloramphenicol [94,97] deserve particular consideration for extraction approaches because of their structural similarity to the analyte. In contrast to many of the other antibiotics, stability data for chloramphenicol samples and extracts are available from several authors [96-99].

The procedures reported for determination of chloramphenicol are largely free of interferences. All of the methods used reversed-phase chromatography with 20-40% CH_3OH or CH_3CN in the mobile phase. The occurrence of some interferences in the precipitation procedures was noted above. The possible interference of metabolites of chloramphenicol has not been rigorously checked but does not appear to pose a problem. The predominant metabolite(s), the glucuronide esters, are much more hydrophilic than even the succinate esters and elute very early. N-deacylated and dehalogenated chloramphenicol metabolites elute early also [92]. Several of the precipitation and the extraction procedures are potentially subject to injected solvent problems.

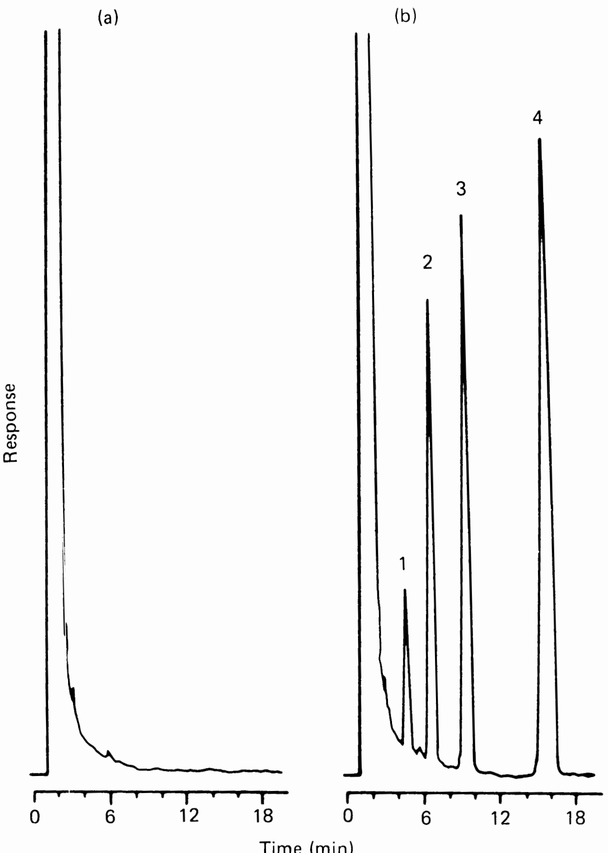

Figure 9 Chromatograms of plasma extracts. (a), blank plasma; (b), plasma containing the following: 4.4 μg/ml chloramphenicol-1-monosuccinate (1); 14.2 μg/ml 3-monosuccinate (2); 13.7 μg/ml chloramphenicol (3); and benzocaine internal standard (4). For conditions, see Ref. 96. (Reproduced with permission of the copyright owner.)

The relative merits of LC and other methods for the determination of chloramphenicol were well summarized by Weber et al. [104]. LC determinations are much better than the microbiological assays to which they frequently were compared, in terms of speed and freedom from interferences. In vitro hydrolysis of the succinate esters is, for example, a potential problem. LC is also preferable to gas chromatography, which requires derivatization of chloramphenicol [105]. The accuracy, precision, and sensitivity of LC were found to be comparable to a radioenzymatic assay [104]. The radioenzymatic assay

required less sample than did the LC determination, but only LC is capable of determining the succinate esters simultaneously. Also it does not require radiolabeled reagents. Thus it is clear why therapeutic monitoring of chloramphenicol plasma concentrations is frequently done by liquid chromatography.

E. Vancomycin

1. Pharmacology

Vancomycin is produced by *Streptomyces orientalis*, an organism which was originally found in soil samples from Indonesia and India. Vancomycin was first isolated in 1956 and was introduced into clinical practice shortly thereafter. The complex glycopeptide structure of vancomycin (see Fig. 10) was not definitively established until many years later. Vancomycin works by inhibition of the synthesis of the cell wall of sensitive bacteria. Vancomycin is active against a variety of gram-positive bacteria and some gram-negative cocci. After the introduction of penicillins and cephalosporins active against penicillinase-producing staphylococci, vancomycin use diminished. More recently there has been a resurgence in interest in vancomycin, particularly in treating methicillin-resistant staphylococci and infections due to *clostridium difficile*.

Vancomycin is primarily administered intraveneously, since it is poorly absorbed after oral administration. The drug is excreted almost entirely by the kidney; the half-life of vancomycin in serum is about 6 hr in patients with normal renal function. Desirable blood levels are on the order of 10-30 μg/ml. Ototoxicity and nephrotoxicity may occur when excessively high concentrations of the drug are present in plasma.

Figure 10 Structure of vancomycin.

Monitoring of blood levels is particularly important in neonates and patients with impaired renal function. Clinical assays of vancomycin are generally performed by microbiological assay.

2. LC Methodology

Two liquid chromatographic methods for the determination of vancomycin in plasma have been reported [106,107]. The procedure of Uhl and Anhalt [106] incorporated ristocetin as an internal standard. Sample cleanup was done by adsorption of the drugs on a weak cation-exchange column. After washing of the column, the drugs were eluted and an aliquot injected. Detection was accomplished at 210 nm, after elution from a reversed-phase column with pH 6 phosphate buffer/9% acetonitrile. This procedure gave good recoveries and was linear and reproducible. McClain and co-workers [107] performed a precipitation of proteins with trichloroacetic acid, and "washed" the supernatant with diethyl ether prior to injection. The ion-pairing reagent, heptanesulfonic acid, was included in their mobile phase, which enhanced the retention of vancomycin somewhat. Their detection wavelength was 280 nm, at which the extinction coefficient for both vancomycin and background interferences are lower. Unfortunately, their chromatography took twice as long as Uhl and Anhalt's procedure (approximately 20 min), despite their lack of inclusion of an internal standard. In addition, background from serum constituents was significantly more of a problem with their precipitation procedure. Both methods appeared to be relatively free of interference from other drugs. This selectivity as well as the speed of these LC assays, makes them advantageous relative to microbiological assays. The relative advantages of these LC methods and the radioimmunoassay available for vancomycin determinations [108] are similar to those discussed above for other antibiotics.

F. Erythromycin

1. Pharmacology

Erythromycin was discovered in 1952 in a strain of Streptomyces obtained from a Phillipine soil sample. The structure of this macrolide antibiotic is depicted in Fig. 11. It is most effective against gram-positive cocci and is useful for a variety of infections, although it is the preferred drug for only a few. The mechanism of action of erythromycin involves inhibition of protein synthesis.

Erythromycin is orally active and is normally administered this way. As the free base, it is formulated as an enteric-coated tablet; the stearate salt is also available. The propionyl or ethylsuccinate esters, which function as effective prodrugs, are commonly used for oral administration. Parenteral administration is also possible. Desirable blood levels for erythromycin are on the order of 1 μg/ml. Serious side effects are only rarely caused by erythromycin.

Figure 11 Structure of erythromycin.

2. LC Methodology

Only one method has been reported for the LC determination of erythromycin in plasma [109]. As with the aminoglycosides, it is not straightforward to develop an LC determination of erythromycin in plasma, since it is polar and does not contain any significant chromophores. In addition, the lower blood levels of erythromycin which must be determined, as well as its relative safety, have mitigated against additional studies of this antibiotic. The procedure reported by Tsuji [109] involved diethyl ether extraction of erythromycin and its ethyl succinate ester away from plasma interferences. Multiple extractions were necessary to achieve good recovery. The ether was then evaporated, the residue reconstituted in mobile phase, and a portion injected onto the LC. After conventional reversed-phase LC, the components were detected by coextraction with a fluorescent ion-pairing reagent (Tinopal). Automation of the extraction required the use of extensive autoanalyzer-like, postcolumn equipment. Other workers have found that this procedure may be extended to erythromycin estolate, with the use of hydroxyerythromycin as an internal standard, but that this approach in general is neither convenient nor routinely reliable [110].

G. Trimethoprim and Sulfonamides

1. Pharmacology

The antimicrobial agent trimethoprim is a derivative of 2,4-diaminopyrimidine. Its structure is shown in Fig. 12. In combination with sulfamethoxazole (or sulfadiazine), trimethoprim is used for treatment of certain urinary and respiratory tract infections. The sulfonamide inhibits formation of folic acid, and trimethoprim is a highly selective inhibitor of the lower organism forms of dihydrofolate reduct-

Figure 12 Structure of trimethoprim.

ase. Since these represent two steps of one overall enzymatic pathway, the two agents act synergistically. There is an optimum ratio of the two for synergism. Sulfamethoxazole has pharmacokinetic properties which are very similar to those of trimethoprim, so that a nearly optimum 20:1 concentration ratio of the two in blood may be obtained and maintained. The combination is administered orally (or parenterally), with blood levels of trimethoprim and sulfamethoxazole peaking within 2 and 4 hr, respectively. Desirable peak blood levels should be between 2 and 40 μg/ml for trimethoprim and sulfamethoxazole, respectively. Both have half-lives of 9-10 hr. The toxicity of this combination is low, so that poor therapeutic response is the most common reason for determination of blood levels. Trimethoprim is generally determined by a fluorometric or microbiological procedure. Sulfonamides can be assayed (but with poor selectivity) colorimetrically or microbiologically.

2. LC Methodology

Liquid chromatographic determinations of sulfonamides alone in plasma have been adequately reviewed by Gerson and Anhalt [2], and only a few recent references [111,112] need to be mentioned in addition to their list.

There have been a number of reports of LC methods for trimethoprim [113-120]. Many of these allow simultaneous determination of sulfamethoxazole (or sulfadiazine) [113,116-118,120], and frequently the N^4-acetyl metabolite of the sulfonamide as well. Unfortunately, from a clinical viewpoint, these assays have primarily been developed for use in pharmacokinetic studies of the drug(s). Most of them involve extraction of trimethoprim (and sulfonamides) into solvents such as chloroform or ethyl acetate. As noted earlier for chloramphenicol, this may not be necessary for therapeutic drug monitoring purposes, where a more simply and rapidly executed protein precipitation approach may suffice. These assays have, as a whole, been little studied in regard to possible interferences and so it is not possible to conclude whether solvent extraction is really necessary.

The chromatographic conditions developed as part of the first reported method for trimethoprim [113] appear to be as good as any reported since. That separation, done on the supernatant of an acid precipitation of proteins, is shown in Fig. 13. The chromatograms depicted in two of the more recent reports were absolutely atrocious [114, 118]. Two other methods [116,120] were based upon normal-phase chromatography, which is probably not a desirable approach (relative to reversed-phase chromatography) for most clinical laboratories. In

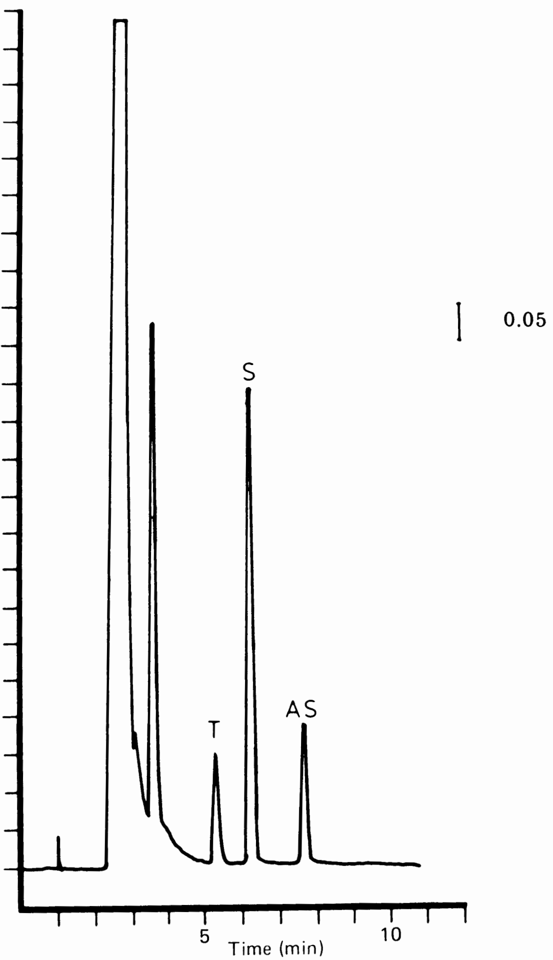

Figure 13 Chromatogram of an extract of plasma which contained trimethoprim (T), sulfamethoxazole (S), and N^4-acetylsulfamethoxazole (AS). For original publication and chromatographic conditions, see Ref. 113.

terms of detection, trimethoprim offers some interesting possibilities. It is of course most commonly detected by UV absorption, but it fluoresces and is electrochemically active. Weinfeld and Macasieb found that fluorescence detection did not increase sensitivity (i.e., absolute detection limits) but it did, of course, increase selectivity [115]. Enhanced sensitivity as well as selectivity may be obtainable by oxidative detection of trimethoprim in an electrochemical detector [119]. In any case, additional work towards an optimal LC assay for therapeutic drug monitoring of trimethoprim is certainly needed.

IV. CONCLUSION

Liquid chromatography has proven to be widely applicable to the determination of antibiotics in body fluids. Workable LC methods have been reported for all but a few of the clinically important antibiotics. LC determinations are sensitive, selective, reproducible, accurate, and convenient. The particular advantage of LC relative to the other widely available techniques for antibiotic determinations is its chemical specificity. On the other hand, there remains room for improvement in current LC methods. Some of the methods reported to date are tedious or difficult. An additional limitation is the lack of procedures capable of determining multiple antibiotics, either without significant procedural modifications, or without any modifications. As noted particularly with the cephalosporins, additional data are needed on the stability of samples under various storage conditions and the stability of sample extracts at ambient temperature. All of these limitations can be expected to be reduced in the future.

A number of instrumental innovations have recently or will shortly become commercially available, which will have an important impact on the utility of LC for antibiotic determinations. One is the simultaneous multiwavelength UV detectors which were recently introduced. These detectors have a linear diode array which is, in essence, a large bank of individual photodiode detectors. The light beam is diffracted after passing through the detector cell. Absorption can then be monitored simultaneously at the range of wavelengths which are spread across the diode array. Multiple-wavelength data allow for spectral differentiation of peaks eluted from the LC. This could be particularly useful, for example, in differentiating cephalosporins which have side chains with varying UV absorption characteristics. The spectra obtained for a peak may be compared to a standard or to a library spectra to assist in confirming the identity of an analyte or identifying an unknown. In addition, the spectra obtained over the course of elution of a single peak may be compared to each other to ascertain the homogeneity of the peak, that is, whether there are two or more compounds coeluting. An excellent demonstration of the potential power of such an approach showed that the presence of as little as 0.1% of a coeluting

compound could be detected [121]. The absolute sensitivity of these multiple-wavelength detectors is not quite as good as the best single-wavelength detector. Nonetheless, because they generate additional information and their cost is reasonable (about the price of two variable-wavelength detectors), simultaneous multiwavelength detectors will find increasing use.

The equipment available for isocratic or gradient LC is rapidly improving, as the engineering of LC systems matures and as microcomputers are increasingly tied into all aspects of the LC equipment. Computers can have a significant impact in three areas: instrument monitoring, data handling, and instrument manipulation. Computers connected to appropriate sensors can monitor the performance of an LC system. When trouble is detected, they can alert the operator and even indicate the likely cause of the problem, simplifying maintenance. This is of particular value where largely unattended operation is desired. In the area of data handling, traditional "integrators" are being replaced by inexpensive yet sophisticated data systems which integrate peaks, perform calculations on the raw data, generate statistics, print appropriate reports, and provide for permanent records of all chromatograms.

The area of computer manipulation or control of LC instruments merits particular discussion. There are several functions which computers can perform in this regard. One very straightforward function is to automatically adjust LC conditions after one set of assays is completed and proceed with additional types of assays. A gradient system is required for this to be done properly, but it greatly increases the versatility of a single LC. With proper development of chromatographic conditions, a simple adjustment of flow-rate, column temperature, or percent organic solvent would suffice for separation of different compounds. A good application would be a system for assaying all or most of a family of antibiotics, such as the aminoglycosides, penicillins, or cephalosporins. Additionally, the computer can evaluate the data from an initial chromatogram and make adjustments to parameters to offset the effects of column age or to try to overcome detected interferences. Computers can also be used to significantly reduce the operator attention needed during methods development. Of particular interest and importance is automation coupled to a systematic approach to methods development. With the increased understanding of the mechanisms of reversed-phase chromatography, it is now possible to optimize separations after only a relatively small number of experiments [122]. Commercial LC systems capable of automated systematic methods development will shortly be available. The use of such techniques and equipment will lead to the availability of versatile and robust LC methods for antibiotics and other analytes in body fluids.

In conclusion, LC has been proven in many laboratories to be an effective tool for therapeutic monitoring of antibiotics. Although many

clinical laboratories currently do not use LC for antibiotic assays, and most of those that do, use LC for only a few of the antibiotics, this situation will undoubtedly change. Due to the inherent power of LC and the impact of better methods and equipment as detailed above, the role of LC in the therapeutic drug monitoring of antibiotics may be expected to increase during the coming years.

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11

ANTIDEPRESSANTS

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I. INTRODUCTION

Depression, the most common psychiatric disorder, may be manifested as a normal state of unhappiness due to a variety of reasons for a short period of time. However, the pathological depressions may last longer, and may relapse with such intensity that would result in the impairment of normal activities [1-3]. Freud [4] defined melancholia, one kind of depression, as follows:

> The distinguishing mental features of melancholia are a profoundly painful dejection, cessation of interest in the outside world, loss of capacity to live, inhibition of all activity, and a lowering of the self-regarding feelings to a degree that finds utterance in self-reproaches and self-revilings, and culminates in a delusional expectation and punishment.

According to a recent study by Boyd and Weissman, the lifetime probability of a major depression reaches up to 12% for men and 26% for women [5]. Annually, affective disorders (depressive or manic) may be experienced by 8–15 million people in the United States [3]. According to the National Institute of Mental Health, the economic cost of depression may exceed 7 billion dollars. Suicidal death related to depression might be estimated to be 80% of 25,000 suicides per year, and is ranked as one of the leading causes of death [1,3]. Thus, depression is indeed a major medical problem [6,7].

Knowing the social impact of depression, this chapter will endeavor to review some basic clinical understanding of depression and to update the monitoring of antidepressants as follows:

1. To review the historical perspectives.
2. To update the various hypotheses and types of depression.
3. To review briefly the clinical pharmacology and pharmacokinetics of the first-generation antidepressants: imipramine, desipramine, amitriptyline, nortriptyline, doxepin (and *N*-desmethyldoxepin), and other antidepressants not presently measured by HPLC, and not used clinically. A detailed treatment will be devoted to the new antidepressants: amoxapine, maprotiline, trimipramine, and trazodone.
4. To delineate the rationale for monitoring, and the role of the laboratory based upon the above discussions.
5. To recommend briefly a sampling protocol.
6. To review reversed-phase HPLC (RP-HPLC) assays of antidepressants from the viewpoints of sample preparation and LC analysis.

II. HISTORICAL PERSPECTIVES

To gain an understanding of the evolution of some of the current hypotheses and treatment of depression, a brief historical account of depressive illness is provided [8]. Early observations by Hippocrates and other physicians led to the identification of depression, with the following symptoms: thought and actions impairments, withdrawal, and suspiciousness. Artaeus (150–200 A.D.), a Greek writer, differentiated between two different kinds of depression, unipolar and bipolar, as defined in modern psychiatric diagnosis. Treatment included bleeding, purgation, etc., to restore humors. During the seventeenth century, depression was explained by acidity disturbance theories, with the possible role of a theoretical black bile as proposed by Hermann Boehaave (1668–1738). This was followed by Friedrich Hoffmann's emphasis on dura mater, resulting in the use of mineral spring water for therapeutic application and in the later introduction of health spas in the western world. Other historical approaches included the emotions attachment proposed by humanist Juan Vives and the vitalism proposed by Georg Ernst Stahl, emphasizing psychotherapy through individualized patient care [8]. The modern treatment of depression has drawn upon the understanding developed in some of these historical observations and "theories," as well as upon the new approach of individualized patient care and changing social, cultural, and economic concerns. Current therapeutic modalities include group and family psychotherapy, electroconvulsive therapy (ECT), and chemotherapy.

The era of using chemotherapy in depressive illness was initiated in 1949 by John Cades, an Australian who treated mania with lithium [9]. Later, lithium was found to be an effective prophylaxis for depression. In the 1950s, major tranquilizers such as phenothiazines were introdued, followed by meprobamate, benzodiazepines, butyro-

phenones, monoamine oxidase inhibitors (MAO-I), and in 1957, iproniazid (a MAO-I) and imipramine (the first tricyclic antidepressant). Kuhn, in his investigation of the neuroleptic properties of phenothiazine-like drugs, observed that imipramine had antidepressant properties. This observation was later confirmed by others. Since then, structurally similar tricyclic antidepressants, such as amitriptyline, doxepin, etc., have been employed. Recently introduced nontricyclics include amoxapine, maprotiline, trazodone, nomifensine, and mianserin. Concurrently with these developments, various hypotheses of depression were being investigated, to gain an understanding of the biochemical cause and the role of various chemotherapeutic agents.

III. HYPOTHESES FOR DEPRESSION

Depression may be explained by the following catecholamine, serotonin, and other hypotheses [10–14]. According to the catecholamine hypothesis proposed by Schildkraut [10–17], depression is due to a functional deficiency of norepinephrine metabolism. The hypothesis is further substantiated by the clinical finding that the inactivation of biogenic amines was increased, due to the fact that reserpine inhibited their binding. Thus, this might have accounted for the depression experienced by hypertensive patients receiving reserpine drug therapy. In animal studies, MAO-I reversed reserpine-produced depression syndromes [17]. The hypothesis further suggests that the major nonrepinephrine metabolite, MHPG (3-methoxy-4-hydroxyphenylglycol), is useful for classifying depression.

The serotonin hypothesis, as proposed by Coppen [18], suggested that the tertiary amines such as amitriptyline and imipramine are potent inhibitors of reuptake of serotonin [18,19]; thus, serotonin concentration increases at the synaptic cleft. Since serotonin is metabolized by monoamine oxidase to form mostly 5-HIAA (5-hydroxyindoleacetic acid), the cerebral spinal fluid HIAA concentration may aid in the selection of secondary amines such as nortriptyline as an effective antidepressant.

According to Maas [20], depression may be subdivided into two categories: (a) low urinary MHPG as a result of a disorder in norepinephrine metabolism for which drugs of choice are imipramine and desipramine; and (b) high or normal MHPG as a result of a disorder in serotonin metabolism, readily treated with amitriptyline. The pharmacological properties of some antidepressants and their effect on the reuptake of biogenic amines are summarized in Table 1, according to Orsulak [21].

Based upon current findings, the role of biogenic amine hypotheses in explaining the action of the antidepressant is inconclusice [14]. Baldessarini favored a search for new antidepressant agents which might not fulfill all of the pharmacological parameters as presently proposed by the biogenic amine hypotheses. For the treatment of

Table 1 Pharmacological Properties of Antidepressants[a]

	Effect on biogenic amine reuptake		Anti-cholinergic activity	Cardio-vascular toxicity
	Norepinephrine	Serotonin		
Tricyclics				
Amitriptyline	0	+++	+++	+++
Imipramine	++	++	++	+++
Nortriptyline	++	+	++	++
Desipramine	+++	0	+	++
Protriptyline	+	+	++	+++
Doxepin	+	+	+++	
Trimipramine	++	++	++	+++
Tetracyclics				
Maprotiline	+++	0	++	++
Amoxapine	++	+	++	++
8-Hydroxy-amoxapine	++	++	?	?
Other classes				
Trazodone	0	+++	0	0
Alprazolam	0	0	0	0

[a]0 = least potent to ++++ = most potent
Source: Reprinted with permission from Ref. 21.

other psychotic disorders, Snyder proposed a dopamine receptor blockage by neuroleptics, accounting for the response of patients suffering from psychotic disorders such as schizophrenia [22].

Birkmayer [23] proposed another hypothesis, stressing the chemical imbalance caused by antiserotonergic and antiadrenergic agents. Endocrinological effects on depression may be related to the dysfunction of the central suppressive mechanisms of the adrenocortical steroid hormones [24]. Thus for patients with endogenous depression, peak and baseline cortisol levels were not suppressed by dexamethasone challenge. However, a later study by Holsboer did not confirm the above finding [25].

Other factors possibly implicated in depression are cyclic adenosine monophosphate (cAMP) and electrolytes. Abdulla and Hamadah [26] proposed that tricyclic antidepressants inhibit the degradation of cAMP, enhancing the return to normal concentration. The clinical application of lithium as antidepressant supports a possibly pivotal role of electro-

lytes in the cause and treatment of depression. Coppen and Shaw [27] proposed that depression may be indirectly related to elevated intracellular sodium.

Thus many hypotheses and explanations have been proposed to elaborate the biochemical cause of depression. Moreover, some drugs, such as antihypertensive and antiparkinson agents may produce some symptoms of depression [3].

For effective treatment, depression may be divided into to major categories [3]: unipolar, that is, single or recurrent major episodes; and bipolar, including both major depressive and manic episodes. Unipolar depression may be further subdivided, according to the Diagnostical and Statistical Manual of Mental Disorders (DSM-III), into five groups:

1. Endogeneous/nonendogenous
2. Psychotic/nonpsychotic
3. Primary/secondary
4. Anxious/agitated/anergic-hypoactive
5. Familial pure depression/depressive spectrum/sporatic depression

Other syndromes that may confound the diagnosis of depression include: anxiety, sleep, obsessive-compulsive, and schizophrenic disorders, anorexia nervosa and bulimia, dementia, sexual dysfunction, dysphoria, and vague somatic complaints. It is important to note that some patients may present a mixture of symptomatology. Through an understanding of the hypotheses and classes of depression, effective treatment modalities and the role of the laboratory, as in the dexamethasone suppression test (DST) and in drug monitoring, may be better defined.

IV. CLINICAL PHARMACOLOGY

For reviews of the pharmacokinetics of tricyclic antidepressants (TCAs), the reader may be referred to the following sources: [28–33]. After oral doses of tricyclics, absorption by the gastrointestinal tract is rapid and complete. Peak plasma levels may be achieved within 2–10 hours. Due to the "first-pass" effect by the liver, the fraction of available drugs is lowered to 13% for doxepin and to 90% for protriptyline. Large interindividual differences exist with respect to pharmacokinetic parameters. Tricyclics are highly bound to plasma proteins, such as albumin, acid α_1-glycoprotein, and lipoprotein, resulting in a large volume of distribution. The free fraction is relatively constant and may be determined by equilibrium dialysis, ultrafiltration, CSF, and saliva measurements. A recent study demonstrated the feasibility of using isoelectric focussing to determine the polymorphic forms of acid α_1-glycoprotein binding with amitriptyline [34].

The metabolic pathways for these drugs involve demethylation, hydroxylation, and glycuronidation, with minor pathways of N-oxidation and dealkylation, followed by renal excretion. Some of the demethylated and hydroxylated metabolites from the major pathways have been shown to be pharmacologically active, and their plasma levels might therefore contribute to the therapeutic response and to side effects. Liver metabolism may be governed by genetic factors, concomitant drug therapy, smoking, weight, race, diet, age, and other factors. Autoinduction of mianserin was recently reported in geriatric patients, requiring dosage readjustments [30]. For patients on concomitant drug therapy, drug interactions include a stimulation by barbiturates and an inhibition by neuroleptics (hydroxylation inhibition). Other medications such as methylphenidate, corticosteroids, disulfiram, and dipropylacetamide also increase TCA levels, possibly due to drug interactions [35].

Due to their lipophilicity, tricyclics readily partition into the tissues and cross the blood-brain barrier. The distribution and elimination may be described by a two-compartment open model: (a) the central compartment (liver, heart, lung, and brain), and (b) the peripheral compartment (blood and other tissues). Elimination half-lives are long, ranging from 6–24 hr (IMI), and 15–93 hr (NOR), to 54–198 hr (protriptyline). Steady-state plasma concentrations may be achieved within 1–2 weeks, as shown by Table 2. These steady-state levels differ significantly [36]. Single oral doses may be used to predict steady-state plasma concentrations [37,38]. Having established the basic clinical pharmacokinetic features of tricyclic antidepressants, the following discusssion reviews other aspects of their clinical pharmacology and pharmacolinetics, with emphases on the role of plasma levels.

A. Imipramine (IMI) and Desipramine (DES)

Imipramine metabolism is shown in Fig. 1. Several clinical studies [28, 30,35,39] demonstrated that the response to IMI or DES is related to their plasma levels. Glassman et al. [40] concluded that plasma levels lower than 150 μg/L correlate with poor response. The response rate increased between 150 and 200 μg/L, "plateauing" around 250 μg/L. Patients with unipolar depression, not responding with levels of 250 μg/L or higher, would not respond with even higher levels, but would suffer from increased side effects. Reisby et al. [41] showed that for patients with endogeneous depression responding to imipramine therapy, favorable response was correlated with IMI/DES levels of greater than 240 μg/L. Simpson [42] recently reported a weak correlation between clinical response and plasma levels of imipramine and desipramine, and advised against monitoring of plasma levels.

Plasma levels of desipramine (29 μg/L to 318 μg/L), i.e., the demethylated metabolites, were correlated with clinical response in two

Table 2 Pharmacokinetic Properties of Antidepressants

	Half-life in plasma (hr)	Peak time oral dose (hr)	Time to steady state (days)
Tricyclics			
Amitriptyline	17-40	2-8	4-10
Nortriptyline	15-93	2-8	4-19
Imipramine	6-24	1-6	2-5
Desipramine	12-76	2-8	2-11
Protriptyline	54-198	6-12	10+
Doxepin	8-36	2-6	2-8
Trimipramine	9	?	2
Tetracyclics			
Maprotiline	27-58	9-16	7-14
Amoxapine	8	1.5	2
8-Hydroxyamoxapine	30	2.1	5
Other classes			
Trazodone	13	1.5-2	3
Alprazolam	12	1	3

Source: Reprinted with permission from Ref. 21.

studies by Khalid et al. [43]. A later study showed that more favorable responses was associated with plasma levels between 107 and 290 μg/L, rather than a low level of 43 μg/L or a higher level of 440 μg/L. Freidel et al. [44] reported that a favorable clinical response was obtained in 15 patients with plasma levels between 37 and 163 μg/L, and indicated diminishing response for levels greater than 160 μg/L. Amsterdam et al. [45] reported that a patient responded to high doses (500 mg/day) with plasma levels of 145 μg/L. Nelson, Jatlow, et al. [46–49] reported that for patients treated with desipramine and neuroleptics, their desipramine levels were elevated, probably due to the inhibition of desipramine metabolism by the neuroleptics, which might be responsible for the increased incidence of toxicity. For patients with nondelusional unipolar depression, the author suggested a threshold of 125 μg/L or higher for effective therapy. Thus a possible correlation of clinical response with plasma levels has been demonstrated in some clinical studies. Further studies will be needed to substantiate this correlation as well as the role of active metabolites in the therapeutic response and side effects.

Imipramine (Active)

Demethylation

Desipramine (Active)

2-Hydroxylation

2-OH-Imipramine (Preclinically Active)

Demethylation

2-OH-Desipramine (Preclinically Active)

Glucuronidization

2-OH-Imipramine Glucuronide (Inactive)

2-OH-Desipramine Glucuronide (Inactive)

Figure 1 Major routes of metabolism of imipramine. (From Ref. 35: *Antidepressants: Neurochemical, Behavioral, and Clinical Perspectives*, S. J. Enna, J. B. Malick, and E. Richelson (Eds.), 1981, p. 188, courtesy of Raven Press, New York.)

Recently, several studies have reported the presence of 2-hydroxy metabolites and possible clinical implications [50—55]. Jandhyala et al. [51] concluded from a study of several TCAs on the hemodynamics and the myocardial contractability in dogs, that both parent drug and metabolites of IMI and DES caused cardiovascular effects and were toxic to the myocardium. Potter et al. [52] showed that the hydroxylated metabolites of IMI, DES, clomipramine (Cl-IMI), and nortriptyline (NOR) blocked the uptake of norepinephrine and serotonin and reversed or prevented the motor retardation and ptosis induced by reserpine. OH-IMI and OH-NOR were identified in rat tissue as well as CSF. A later clinical study of the steady-state plasma samples from both young and adult patients showed that the ranges of the OH-IMI/IMI

ratio and the OH-DES/DES ratio were 0.22–0.27 and 0.46–0.58, respectively [53]. These findings were confirmed by DeVane and Jusko [54]. Thus, the concentrations of parent drugs and metabolites (such as the hydroxylated metabolites) were substantial and showed great variability. Nelson et al. [55] reported that 2-OH-DES plasma levels, with or without including DES levels, were not correlated with clinical response and side effects; thus these levels would not be useful in monitoring the treatment of depression. Further studies are needed to validate the role of hydroxy metabolites in the therapeutic response and side effects.

B. Amitriptyline (AMI) and Nortriptyline (NOR)

The metabolic pathways are outlined in Fig. 2. Like imipramine, the demethylated and hydroxylated metabolites of these agents have been demonstrated to be pharmacologically active. However, published clinical studies have shown that the correlation of AMI plasma levels with clinical response have been inconclusive. Braithwaite et al. demonstrated the plasma level/response curve to be linear [56]. A lower limit of 120 μg/L for combined AMI and NOR was indicative of less than favorable response. Montgomery [57] later showed a curvilinear relationship between the plasma concentrations of amitriptyline and therapeutic response. For patients with endogenous depression, response was observed at plasma levels less than 120 μg/L, but the rate of response increased with increasingly high concentrations. Kupfer [58] concluded that total levels greater than 200 μg/L correlated with the best response. However, a multicenter European study by Coppen et al. [59] did not confirm such correlation. A similar observation was made by Robinson [60]. In summary, in treating endogenous depression there seems to be a correlation between response and plasma drug levels. Further studies which consider plasma levels of hydroxylated metabolites will be needed to establish such relationships.

In contrast to AMI, NOR (i.e., its demethylated metabolite) has been subjected to less controversy. Asberg et al. [61] demonstrated a good correlation of clinical response with plasma levels between 50 and 150 μg/L. This has been substantiated by later studies by Montgomery et al. [62]. Levels of 150 μg/L have been associated with decreased response and side effects.

C. Doxepin

Doxepin is metabolized via demethylation to N-desmethyldoxepin. Several studies examined the relationship of total plasma levels to clinical response. Kline [63] concluded that a desmethyldoxepin (not doxepin) level of 20 μg/L or more was correlated with clinical response. Friedel and Raskind [64] showed that a total level of 110 μg/L or more was

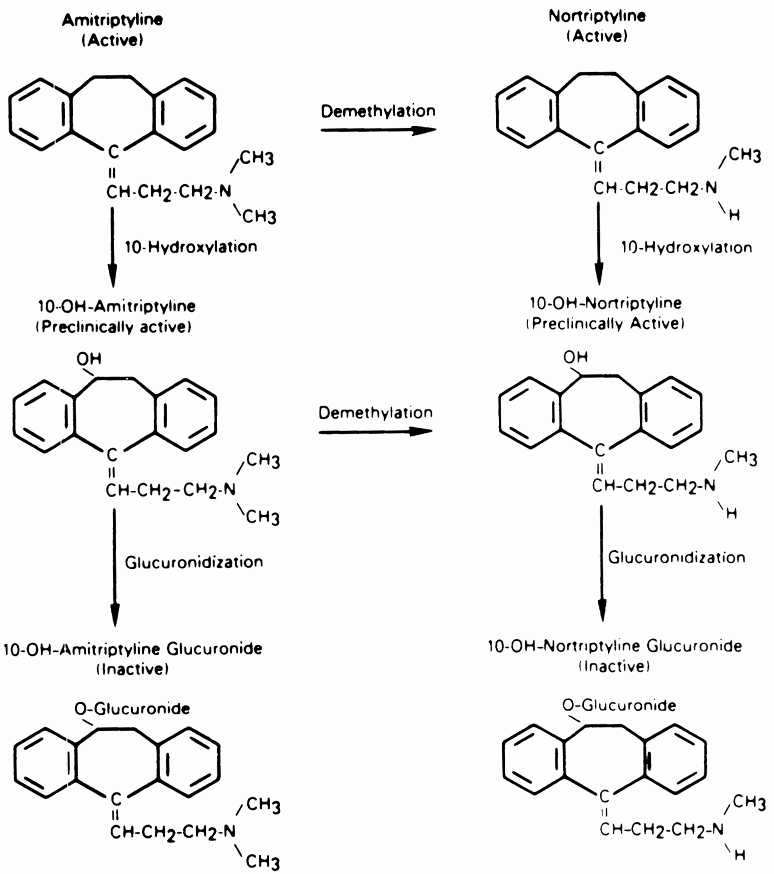


Figure 2 Major routes of metabolism of amitriptyline. (From Ref. 35: *Antidepressants: Neurochemical, Behavioral, and Clinical Perspectives*, S. J. Enna, J. B. Malick, and E. Richelson (Eds.), 1981, p. 188, courtesy of Raven Press, New York.)

needed for response. Green [65] proposed a therapeutic range of 150–250 μg/L, based upon a case report. Jobson [66] studied the effects of smoking and diet on plasma levels versus clinical response. It can be concluded that for treating endogenous depression, there seems to be a relationship between total plasma levels of doxepin and of N-desmethyldoxepin and clinical response.

D. Amoxapine (AMO)

Amoxapine, recently approved for clinical application, has been shown to have a fast onset of therapeutic response with minimal anticholinergic and cardiotoxic side effects [67–69].

AMO is structurally similar to imipramine and to loxapine, a neuroleptic. Pharmacokinetic studies have shown that after rapid absorption, AMO is hydroxylated by the hepatic microsomal enzyme system to form 7- and 8-hydroxyamoxapines (7- and 8-OH-AMO), as shown in Fig. 3 [70,73]. 8-OH-AMO, an active metabolite, accumulates to a significant plasma concentration. A recent report showed the possible existence of a "therapeutic window" for AMO and 8-OH-AMO with clinical responses, as indicated by both Hamilton and Beck rating scales [71]. Significant amounts of AMO and 8-OH-AMO were also identified in serum of an AMO patient who developed galactorrhea [72]. Furthermore, the structural similarity of AMO to loxapine suggested that AMO and its metabolites may possess some neuroleptic activity. Indeed, neuroleptic-like side effects were observed in some AMO patients. Thus the measurement of AMO and its hydroxylated metabolites may aid in the further understanding of its clinical pharmacology. In the author's study [73] of seven outpatients treated with AMO (150–225 mg daily doses), the following plasma levels were obtained: AMO, mean = 89 and range = 40--216 μg/L; 8-OH-AMO, mean = 210 and range = 131--308 μg/L; while 7-OH-AMO levels were either small or not detected, range = 0–13 μg/L. Recently, neuroleptic side effects of AMO were noted in a case report, suggesting the possible role of 7-OH-AMO [74]. This interesting finding and the availability of an assay for 7-OH-AMO may aid in understanding the contribution of 7-OH-AMO to the neuroleptic-like side effects in certain AMO patients.

E. Maprotiline (MAP)

Maprotiline, a tetracyclic antidepressant capable of producing fast onset of response, has been recently approved for clinical application. Its three-dimensional, nonplanar structure may be partly responsible for the selectivity by the receptor sites. MAP therapy is associated with minimal anticholinergic and cardiotoxic side effects [69,76].

(a) (b) (c)

Figure 3 Hydroxylation of AMO: (a), amoxapine, (b) 7-OH-amoxapine, (c) 8-OH-amoxapine. (Reprinted with permission from *Clinical Chemistry*, Ref. 73.)

MAP is demethylated to form *N*-desmethylmaprotiline (ND-MAP), as shown by Fig. 4, and undergoes minor oxidative pathways to form phenolic metabolites [75]. Miller [76] and Matussek [77] reported a lack of correlation of plasma levels of MAP with clinical response and with side effects. Even though substantial amounts of both MAP and ND-MAP were detected in the patient's serum, ND-MAP was not included in the correlation studies.

Schatzberg et al. reported that MAP therapy was more effective for patients with low pretreatment urinary levels of 3-methoxy-4-hydroxyphenylglycol (MHPG) [78]. Mean MAP plasma level of this group was 185 μg/L; therapeutic range was proposed to be 180–400 μg/L. A recent study by the author [73] showed that in seven outpatients treated with MAP (150–225 daily dosage), the following plasma levels were obtained: MAP, mean = 291 and range = 72–494 μg/L; ND-MAP, mean = 96 and range = 42–222 μg/L. These levels compared favorably with previous studies as shown by Table 3 of that paper. The relationship of MAP and ND-MAP with clinical response and side effects awaits further studies.

F. Trimipramine (TRI)

Trimipramine is another recently introduced antidepressant. Its metabolism is similar to that of imipramine, primarily demethylation and possibly hydroxylation of the tricyclic ring. Settle and Ayd [79] found TRI to be as effective as all other antidepressants, with the possible additional benefits of anxiolytic and sedative effects. Presently, there are no data on the correlation between plasma level and response. The author's limited experience in two patients [80] demonstrated plasma levels to be 500 and 800 μg/L. A substantial amount of *N*-desmethyltrimipramine was also detected.

G. Trazodone (TRA)

Trazodone is a recently approved antidepressant. Unlike first-generation antidepressants such as amitriptyline, but similar to second-generation antidepressants such as maprotiline and amoxapine, TRA may elicit a fast onset of response [69,81–84]. TRA is claimed to lack

(a) (b)

Figure 4 *N*-desmethylation of (a) maprotiline to (b) *N*-desmethylmaprotiline. (From Ref. 73.)

anticholinergic side effects and cardiovascular toxicity [85,86]. However, a recent report showed that TRA might cause ventricular ectopy [87]. Current literature indicates that TRA has been well tolerated by overdose patients [81,88].

TRA, classified as a triazolopyridine (Fig.5), is considered to be a broad-spectrum antidepressant [89]. It inhibits serotonin reuptake but does not potentiate catecholamines or inhibit monoamine oxidase. Its clinical efficacy was compared to impiramine, desipramine, clomipramine, and amitriptyline. Pharmacokinetic studies [90–95] showed that TRA is rapidly absorbed, and peak plasma concentration may be achieved within 1–2 hr. Metabolism is extensive, mainly via hydroxylation and oxidation. Elimination is biphasic, with half-lives of 4.4 hr and 7–8 hr for the two respective time periods of 3–10 hr and 10-34 hr post-ingestion. Plasma TRA concentrations of fasting patients are higher than those of nonfasting patients. Therapeutic and toxic concentrations have not been established. In a recent study by the author [96], the plasma concentrations of 26 patients treated with TRA (50–300 mg/day) were mean, 639 and range, 73–1678 μg/L. Two geriatric patients on 300 mg daily dose achieved levels of 1983 and 1938 μg/L, while levels of two overdose patients were much higher, about 5000 μg/L, as shown in Fig. 6. Correlation of TRA with clinical response awaits further studies.

H. Mianserin (MIA)

Although mianserin is used only for clinical research in this country, it has been used clinically for treating depression and depression associated with anxiety in Europe, Australia, and New Zealand [97–101]. MIA, a tetracyclic antidepressant, is classified as a dibenzopyrazino-

Figure 5 Chemical structures of (a) trazodone (TRA); (b) trazodone internal standard (TRA-IS); and (c) mianserin (MIA). (Reprinted with permission from *Clinical Chemistry*, Ref. 96.)

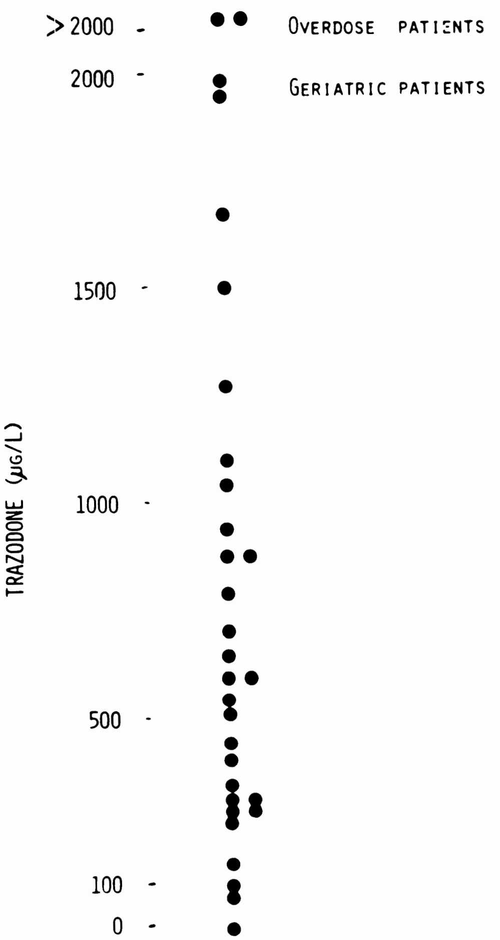


Figure 6 Plasma TRA concentrations of patients receiving 50–300 mg/ daily doses (n = 29) and of two overdose patients.

azepine (Fig. 5). It has low anticholinergic and cardiotoxic side effects, and it increases the turnover of brain noradrenaline [99,100]. In animals, it does not affect central serotonin uptake. Its clinical efficacy was compared to that of amitriptyline, imipramine, and clomipramine. Pharmacokinetic studies [101–103] have shown that MIA is absorbed rapidly, achieving peak plasma concentrations in about 2 hr. Metabolism is extensive, mainly through aromatic hydroxylation, N-oxidation, and N-demethylation [8,17]. Elimination half-life is 17 hr. Therapeutic range was proposed, by Montgomery [98] to be 15–70 μg/L, but a recent study by van der Veen [103] did not substantiate such findings.

However, Altamura [101] showed that plasma concentrations correlated with response for involutional and primary depressions, and recommended monitoring of MIA plasma concentrations for checking noncompliance of patients during long-term therapy.

I. Monoamine Oxidase Inhibitors (MAO-I)

Historically, the first MAO-I, iproniazid, was introduced at the same time as imipramine, in the early 1950s. As a group, these drugs are less important than the TCAs, but are indicated when TCAs are ineffective. MAO-I may be useful for atypical depression, such as neurotic depression, anxiety, and phobias. Due to their toxicity, drug interactions, hypertension, and other side effects, MAO-I are less popular. In fact, iproniazid has been banned from some countries. MAO-I may be classified according to chemical structure into two groups: (a) nonhydrazine, including iproniazid, isocarboxazid, and phenelzine (the most widely used): and (b) hydrazine, including tranylcypromine and pargyline [105].

The basis for MAO-I pharmacological actions is the inhibition of monoamine oxidase. The blocking of the deamination within the aminergic neurons results in an overall increase in amine levels. This has been proven to be questionable, however, and current understanding emphasizes the different forms of the monoamine oxidase enzyme. Thus, MAO-I may be defined as type A, if norepinephrine and serotonin are the substrates, or type B, if phenylethylamine and benzylamine are the substrates [106]. After oral administration, MAO-Is are nearly totally absorbed, and maximum inhibition may be achieved within 5–10 days. Clinical response may persist for 1–3 weeks. Information on plasma levels and their relationship to response and side effects is lacking, probably due to low-level and irreversible binding to enzymes [107]. Available information does not support a need for monitoring plasma levels. Presently, there is revewed interest in developing MAO-Is without the undesirable side effects.

J. Lithium

Lithium, initially used for psychiatric excitation, is an effective antidepressant and antimanic agent in some patients [108–110]. Initially, toxicity and side effects were reported [111]. Then in the early 70s, lithium was accepted by the American psychiatric community and is now used in the treatment of bipolar disorder, manic-depressive illness, as prophylaxis in recurrent depression, and in investigations of other disorders. The mechanism of action of lithium therapy is unknown. It does not block biogenic amine reuptake and does not inhibit monoamine oxidase. Thus its action may differ from the postulated modes for TCAs and MAO-I.

Routine lithium monitoring is indicated for adjusting dosage and avoiding toxicity, due to its narrow therapeutic range (0.6–1.2 mmol/L) and low therapeutic index (1.5 mmol/L). Further studies will be needed to understand its mode of action.

K. Other Antidepressants

Stimulants, such as *d*-amphetamine, methamphetamine, and methylphenidate, have been used as antidepressants. Due to possible abuse, side effects, and questionable effectiveness, they are not widely used as antidepressants today. Metabolites such as L-tryptophan, and *S*-adenosylmethionine were used as experimental medications for the treatment of depression.

V. RATIONALE FOR ANTIDEPRESSANT MONITORING AND THE ROLE OF THE LABORATORY

The rationale is similar to that for more estabilished groups of drugs, such as antiepileptics, and has been reviewed extensively [28,30,32, 33,39,112]. In order to correlate clinical response to plasma drug levels, the receptor binding of the drug must be reversible. Free drug concentrations may be governed by the law of mass action. In some of the pharmacokinetic studies discussed in the previous section, drug levels did not correlate with dosage. These studies also showed that wide interindividual variation (20- to 40-fold) of plasma concentrations exist for some patients on the same dosage regimen. This variation may be due to individual drug metabolism which is influenced by protein binding, concomitant drug therapy, and various states such as renal or hepatic disorders, alcohol, smoking, etc. Since TCAs, in general, have a delayed onset of action and may cause side effects, rational utilization of plasma levels may be a useful guide to achieve the desired therapeutic benefits. Thus drug measurement can ensure that the patient plasma level may be titrated accordingly. Since TCA therapies are characterized by steep concentration-effect curves, monitoring may also be helpful for patient management as in the case of nortriptyline. Based on the various studies related to classification of depressions, it may be possible to prescribe the appropriate TCA according to a patient's urinary MHPG level or the result of a dexamethosone suppression test (DST). Noncompliance may be easily checked by measuring TCA plasma levels of nonresponders. Finally, toxic side effects may be explained on the basis of high drug plasma levels.

As emphasized in Chap. 2, the plasma level is a helpful adjunct for clinicians, but it should *never* be taken as the sole criterion. Thus the role of the laboratory is *supportive* and *interactive*, supportive in the sense that the measurement should be rapidly performed using the established procedures with good precision. TCA measurements by com-

mercial laboratories have been questioned [113]. A recent TDM survey (April, 1983) by the College of American Pathologists showed that only 74 of 1042 laboratories measure nortriptyline. This underscores, in part, that for some labs, technical problems exist. With the reported lowering of drug concentration levels due to a plasticizer of some blood collection vacutainer tubes having been established [114], Narayanan et al. [115] have attempted to identify a suitable blood collection device, as outlined in Chapter 4. Similar studies should be carried for blood collection of new drugs. Realizing the current confusing status of the interpretation of TCA plasma levels, laboratories knowledgeable in pharmacokinetics should interact and attempt to communicate with psychiatrists in the event of abnormally high or low results, keeping in mind the possibilities of drug-drug interaction, overdose, and other considerations. Through a combination of common sense, active and caring communication, TCA plasma levels could become increasingly used by the clinician to ultimately benefit the patient's treatment of depression.

VI. SAMPLING CONSIDERATIONS

Blood collection and related problems have been well documented [114], and systematic studies have since shown that certain new commercial blood collection devices have acceptable clinical efficacy [115]. Some of these areas have been explored in the chapter by Narayanan and Lin (Chap. 4). Briefly, a palsticizer, tris(2-butoxyethyl)phosphate (TBEP), and heparin altered the binding of antidepressants to plasma proteins, producing spuriously low plasma drug levels [116]. These observations point out the need for careful consideration of blood collection devices for new drugs.

Another consideration is the type of specimen, the most common ones being plasma, serum, or whole blood. Due to interindividual differences in the distribution of some drugs between plasma and erythrocytes, it has been suggested that pharmacokinetic studies be performed using whole blood levels. Based upon published data and our own experience of specimen storage, whole blood samples may be kept at room temeprature for up to 24 hr. If possible, plasma or serum should be prepared and kept frozen. These specimens are stable up to 120 days.

Finally, to correlate levels with clinical response, steady-state trough levels (five elimination half-lives) should be measured by performing blood drawing in patients about 15–30 min before the next dose. If the patient is on nighttime dose, the sampling may be done approximately 12 hr post-ingestion.

VII. ANTIDEPRESSANTS ASSAYS

A. Literature Review of Liquid Chromatographic Methodologies

Scoggins et al. recently reviewed TCA monitoring up to 1978 [29,117]. Since then, the reversed-phase mode had been used in the majority of the published studies as shown by Table 3. An important advance, the "FAST-LC," was developed by Snyder et al. [125] using automated sample preparation through liquid/liquid extraction and automated analysis. Readers are encouraged to refer to that original article for important details and Fig. 6 of Chap. 3 for the schematic. Within the area of sample preparation, commercially available extraction tubes such as Bond Elute, Sep-Pak, and others are increasingly used for drug assays. Another recent development has been the introduction of the "second generation" antidepressants such as trimipramine, amoxapine, maprotiline, and trazodone. Their clinical pharmacology and pharmacokinetics have been outlined in a previous section. Further clinical studies will be needed to examine the role of these new drugs and their metabolites, such as the hydroxylated metabolites. In summary, since the reviews by Scoggins and others, great strides have been made so that clinical monitoring of TCAs by RP-HPLC is readily and easily adaptable to the clinical laboratory without presenting major difficulties.

B. Preliminary Assay Considerations

Antidepressant monitoring by clinical laboratories at present is limited to about 74/1094 or 6.8%, according to a recent survey by the College of American Pathologists [136]. Previously, the major limitations were the instrumentation (either GC or HPLC), the availability of easily adaptable published methods, the low antidepressant concentrations (25–300 μg/L), and the questionable clinical demand. It is the author's opinion that the first three factors are no longer limiting, as evidenced in the surveys and published literature reviewed in the previous section. The fourth limitation remains, as more clinical research and pharmacokinetic data become available. The most important guideline for successful clinical antidepressant measurement is *simplicity*, in order to insure precision and sensitivity.

In designing a simple antidepressant assay, there are two interrelated, major considerations: chemical and clinical. Antidepressants, mainly tricyclic and tetracyclic and a few nontricyclic chemicals, are lipophilic bases and thus "sticky" by nature. Thus for extraction into organic solvent, alkalinzation is the first step. Depending upon the requirement of the procedure, such as GLC, further multiple steps, such as extraction and/or derivatization may be needed. On the other hand, for HPLC procedures, the extracts/organic phase may be evaporated or back-extracted by dilute acid. Inherently, HPLC sample prep-

Table 3 LC Assays of Antidepressants[a]

Drugs [Ref.]	Extraction solvent (s)	LC mode	Detection (nm)	Analysis time (min)	CV%	Sensitivity
AMI, NOR, IMI, DES [118]	*n*-Hexane IAA	Normal (Silica B-5)	211	14	5-8	10 μg/L
AMI, NOR + metabolites [119]	*n*-Hexane/dil. H_2SO_4 + NaOH/ diisopropyl ether	Ion-pair (Partisil 5)	254	12	5-10	25 μg/L
AMI, NOR, IMI, DES, DOX [120]	*n*-Hexane + IAA/ dil. HCl	Ion-pair (μBondapak C_{18})	254	13	2-7	2 μg/L
AMI, NOR, IMI, DES, DOX, ND-DOX, PRO [121]	Clin-Elut^{TM}/dil. HCl + MeOH/hexane/butyl chloride	Reversed-phase (Spherisorb S5CN)	210	6	3-9	25 μg/L
AMI, NOR, DES, IMI metabolites [122]	*n*-Hexane + IAA/ dil, perchloric acid	Reversed-phase (μBondapak C_{18})	254	14	6-8	5 μg/L
AMI, NOR, IMI, DES, DOX, ND-DOX, TRI, MIA, AMO, MAP, ND-MAP, TRA, MIA [73, 80, 96, 123]	*n*-Heptane or *n*-hexane + IAA/ dil. H_3PO_4	Reversed-phase (μBondapak C_{18})	254/214	9-14	3-8	0.5-5 ng

Table 3 (Continued)

Drugs [Ref.]	Extraction solvent (s)	LC mode	Detection (nm)	Analysis time (min)	CV%	Sensitivity
2-OH-DES [124]	CH_2Cl_2 + IAA/ phosphate	Reversed-phase (ODS-2)	254	14	5	3 ng
7- and 8-OH-AMO [73]	Ethyl acetate/ phosphate	Reversed-phase (MC-18)	214	22	3-5	1 ng
AMI, NOR, IMI, DES, DOX, PRO [125]	"FAST-LC" Isooctane/1-propanol/dil. H_2SO_4	Reversed-phase (C_8)	205	8	5-8	5 μg/L
IMI,DES [126]	*n*-Hexane + IAA/ dil. H_3PO_4	Reversed-phase (μBondapak phenyl)	252 Ex 360 Em	15	2-15	1 μg/L
AMI, NOR, IMI, DES [127]	MeOH + EtOAc hexane/dil. HCl	Reversed-phase (Micropak MCH-10)	210	14	4-8	<25 μg/L
OH-AMI, OH-NOR (cis and *trans*) [128]	*n*Hexane + IAA/ dil. HCl	Reversed-phase (C6 Spherisorb S5)	Far UV	—	5	2 μg/L
IMI, DES, AMI, NOR [129]	*n*-Hexane + IAA/ dil. acid	Reversed-phase (μBondapak C_{18})	215	11	<7	<25 μg/L
AMI, NOR, IMI, DES, DOX, ND-DOX [130]	*n*-Hexane + IAA	Normal phase (Varian SI-10)	254	8	2-10	15 μg/L

IMI, DES, 2-OH metabolite [131]	*n*-Hexane + n-butyl alcohol	Normal phase (Silica B-5)	240 Ex 370 Em	21	2-4	1 ng
AMO, 8-OH AMO [132]	Clin-Elut™/1-butanol + hexane/dil. HCl/1-butanol + hexane	Reversed-phase (μBondapak C_{18})	254	7	6-12	50 μg/L
TRI, DOX, ND-DOX, AMI, NOR, IMI, DES, MAP, PRO [134]	Manual: *n*-Hexane Automated: Prep-1 Type W cartridge	Reversed-phase (μBondapak CN)	254 254	13 13	9-13 5-9	10 μg/L 5 μg/L
AMI, NOR, and OH metabolites [135]	*n*-Hexane + IAA	Normal-phase (Lichrosorb SI60)	240	9	2.4-10.2	2.5-5 μg/L
AMI, NOR, DOX, ND-DOX, IMI, DES, PRO, AMO, 8-OH-AMO, MAP, ND-MAP [137,138]	Bond-Elut™ C_{18}/methanol + ammonium acetate	Normal phase (μPorasil or silica-Supelco)	214-254	12	1.8-18.8	2-15 μg/L
AMO, 8-OH-AMO, MAP [139]	*n*-Hexane + EtOAc or *n*-Hexane + IAA	Reversed-phase (Supelcosil-CN)	211	11	2-5	10-25 μg/L

[a]Nonstandard abbreviations: IAA, isoamyl alcohol; AMI, amitriptyline; IMI, imipramine; DES, desipramine; NOR, nortriptyline; DOX, doxepin; ND-DOX, *n*-desmethyldoxepin; MAP, maprotiline; ND-MAP, *n*-desmethylmaprotiline; PRO, protriptyline; MIA, mianserin; AMO, amoxapine; 7- and 8-OH-AMO, 7- and 8-hydroxyamoxapine; TRI, trimipramine; Ex, excitation wavelength in nm; Em, emission wavelength in nm; EtOAc, ethyl acetate; and TRA, trazodone.

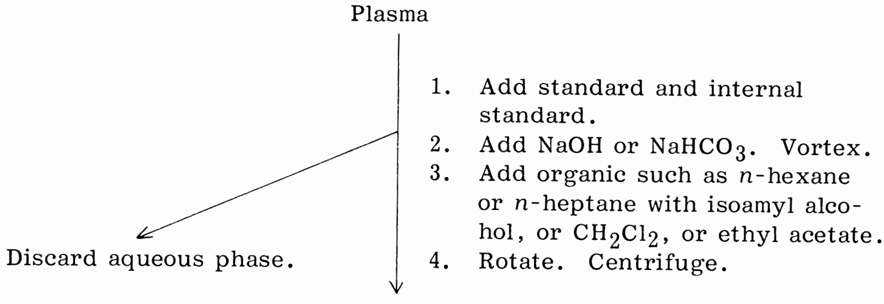
aration is simpler than GLC. (Due to the high pK_as of these lipophilic bases, Vandemark described a novel separation using alkalinized mobile phase to minimize tailing [118].) Due to the "sticky" nature of these drugs, adsorptive loss may be minimized by using deactivated surfaces such as silanized or polypropylene tubes. If possible, disposable tubes should be used to avoid cross-contamination.

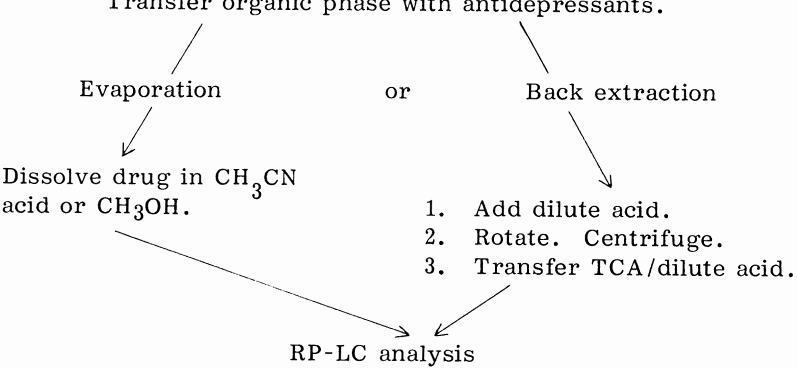
Clinical considerations include the concentration range, the matrix of the specimen (serum or plasma), the stability of stock solution, and the ease of sample preparation and analysis. In considering noncompliance and overdose cases, and in the experience of our laboratory, a useful assay range lies between 25 and 800 or 1000 μg/L. For pharmacokinetic studies, lower limits down to 5 μg/L are desirable. Using a well-established clinical assay with appropriate reduction in the amount of internal standard, pharmacokinetic measurements may be performed. In retrospect, the low concentration had imposed severe limitations on the extraction and the possibility of obtained measurements with acceptable precision and accuracy. Depending on the matrix (i.e., mostly serum or plasma) and the extraction/analysis procedures, a laboratory would have to determine the specimen of choice in order to enhance sample collection and to avoid possible interfering "ghost" peak. From the author's experience, plasma (using lavender Vacutainer Tubes containing EDTA) provides satisfactory results. The plasma sample volume ranges from 750 μl to 2 ml, depending on the method. The stability of the drug stock solution and the preparation of a calibration standard with "drug-free" plasma (checked by the "blank" chromatograms) should be established as follows: The drug standard solutions, prepared mostly in aqueous solution, may be kept inside siliconized tubes at 4°C up to 3 months. "Drug-free" plasma, or more accuratley, "noninterfering" plasma, is more difficult to obtain and each batch should be analyzed to ascertain noninterference. (Under desperate circumstances, the author and his laboratory personnel have volunteered to be drug-free blood donors!)

C. Sample Extraction

Sample preparation may be divided into three major categories: (a) liquid/liquid; (b) liquid/solid [121] utilizing commercially available extraction columns; and (c) semiautomated [134] and totally automated analyses, using QAI and "FAST-LC" [125]. The majority of the published procedures have utilized the first extraction mode. Thus this section will describe in detail such an approach from the author's experience. Scheme 1 depicts the process of antidepressant sample preparation.

In order to minimize drug adsorption, use silanized glassware (test tubes and pipette) or polypropylene. Recently. a large amount of maprotiline was used to minimize adsorption for the extraction of





Scheme 1 Antidepressant sample preparation.

AMI, NOR, IMI, and DES [135]. Since maprotiline is used clinically, the assay's application would be limited.

Prepare primary methanolic drug stock solutions (1 gm/L) in a silanized volumetric flask. Keep at −20°C. Use a silanized pipette, such as SMI, to prepare working stock solution.

The working stock solution (10 μg/L) may be prepared with water, methanol, or plasma, depending on the drug stability. For example, for first-generation TCAs such as AMI or IMI, working aqueous stock solutions are stable up to three months; however, trazodone dissolved in plasma, but *not* the aqueous or methanolic stock, exhibits similar stability.

Calibration standards are prepared by adding aliquots of these working stock solutions, with the range depending upon the drug, for example: 0−1000 μg/L for AMI, IMI, MAP, and AMO; 0−2000 μg/L for TRA; and 0−200 μg/L for MIA. Then an internal standard, a chemical with similar structure, is added, eliminating the effect of extraction variations. For most tricyclic and tetracyclic assays, the internal standard is clomipramine, an investigational drug in Europe, or protriptyline, a seldom used antidepressant. Since trazodone struc-

ture differs significantly from TCAs, another internal standard (TRA-IS) is used, as shown in Fig. 5.

Add alkaline solution such as NaOH or $NaHCO_3$, and an organic solution/mixture such as *n*-hexane/IAA, or *n*-heptane/IAA, ether, CH_2Cl_2, or ethyl acetate. Extraction is carried out by either shaking or rotation. Centrifuge to separate the organic and aqueous phases. Transfer the organic phase with silanized glass or polypropylene pipettes to another set of extraction tubes. If the drug is not readily extracted by dilute acid (total recovery less than 60%), such as in the case of trazodone, evaporation and reconstitution with dilute acid or methanol should follow. The volume of the dilute acid, or methanol should be 200 µl or more, because of (a) the partitioning loss, (b) the need for sufficient volume for easy transfer, and (c) the multiple injection of 50 µl for high drug concentration measurement. Alternately, for drugs such as AMI or IMI, back extraction with dilute phosphoric acid should be used, since recovery exceeds 60%. If the aqueous phase settle down at the bottom, use a silanized pipette, and with positive air bubbling, introduce the tip to the aqueous layer often existing as a big droplet due to the difference in surface tension. Suction the "droplet" into the pipette with a small amount of the organic phase. Remove the pipette from the test tubes. Bubble the solution further by suction to separate the lower aqueous layer from organic phase. After drying the transfer-pipette wall, introduce the lower layer into another small conical tube, leaving a small amount with the orgainic phase to avoid contamination. The extraction process is readily completed within 1 hr. The extracts in dilute acid or methanol are stable up to 24 hr.

D. Reversed-phase Liquid Chromatographic Analyses

Antidepressant monitoring by LC is mainly performed using the reversed-phase mode, as shown by Table 3, collected from published studies. This section will deal with the optimization of chromatographic parameters for reversed-phase separation, based largely on the author's own experience and selected literature.

The advantage of choosing reversed-phase for analyzing TCAs have been thoroughly discussed elsewhere. Briefly, the major considerations are the stability of the packing, the reproducibility of the analysis, the rapid equilibration, the wide column selection and the compatibility with biological sample matrix.

Recently published assays have utilized mainly C_{18} columns and some CN and phenyl columns. However, the long-term stability of CN columns awaits further investigation. Based upon premises adopted by McHugh and co-workers [140], phenyl column stability may be longer than that of CN, but shorter than that of C_{18}. Both CN and phenyl columns offer minimum tailing, shortened analysis time, and

ambient temperature separation. Further studies are needed to examine the use and stability of CN and phenyl columns for TCAs and other analyses.

Using the C_{18} column to separate imipramine and desipramine, the carbon load (that is, the carbon content of column packing) would be about 10%. However, for the separation of the hydroxylated metabolite (e.g., for 2-OH desipramine and 2-OH imipramine), a higher carbon load of 15% is needed. For a more difficult separation, such as for 7-OH and 8-OH amoxapine, a 5-μm, 30-cm 20% carbon load is needed, with correspondingly high back-pressure (e.g., 300–350 bar). For such a column, in order to maintain a small HETP (height equivalent to the theoretical plate, an efficient separation), the flow-rate should be about 1.5 ml/min.

The percentage of organic modifier such as acetonitrile, methanol, and THF would range from 20 to 40% in order to resolve the metabolites (hydroxylated and demethylated) from parent drugs. A high percentage is desirable to maintain peak symmetry and to hasten elution. The salt concentration may be used effectively, with high concentrations up to 0.2 M employed to enhance peak symmetry; probably as a result of suppressed interaction of the drug with the silanol groups. The pH of the aqueous mobile phase may be maintained at about 4–5.

To enhance peak symmetry and complete analysis with capacity factor less than 10, elevated temperatures of 50 or 60°C have been used without noticeable column degeneration. It is important to pressurize the column prior to heating, and during cooling down to avoid column voiding.

The detection mode of choice is UV, with setting at either 214 or 254 nm, depending on the drug and the sensitivity required. Both electrochemical and fluorescence detectors may provide unique selectivity and sensitivity for monitoring low concentrations.

In order to avoid "salting-out" of the column and chromatograph after each analysis, the column should be cleaned by using 40 ml of a mixture of water and acetonitrile (6:4). Finally, in order to prolong the column life, guard columns should be used and packing changed regularly, e.g., bi-weekly for frequently used columns. With the judicious choice of column parameters as recommended above, antidepressant monitoring by RP-HPLC may be carried out easily.

Table 4 summarizes the chromatographic parameters from the author's experience. Figures 7 through 12 show examples of chromatograms of plasma extract containing antidepressants and metabolites. The linearity, precision, and recovery have been checked and are shown to be acceptable. The reader may be referred to the original publications. Figure 13 shows the calibration curve for MAP and ND-MAP. Briefly, the peak height ratios are linearly correlated to drug concentration between 25 and 1000, or 2000 μg/L for different drugs. Precision studies show that with-in run and between-run CVs are less than 5 and 10%, respectively. Recovery range was from 60 to 80%.

Table 4 RP-HPLC Parameters for Antidepressants Monitoring

Methods	A	B	C	D
Drugs	AMI, NOR, IMI, DES DOX, ND-DOX, MAP, ND-MAP, TRI, AMO, TRA, MIA	AMI, NOR, IMI, DES, DOX, ND-DOX	2-OH-DES 2-OH-IMI	7-OH-AMO 8-OH-AMO
Column and particle size	μBondapak (4.6 mm × 30 cm) 10 μ	Two mini phenyl column (4.6 mm × 5 cm), 5 μm	ODS-2 (4.6 mm × 25 cm) 10 μm	MC-18 (4.6 mm × 30 cm) 5 μm
Carbon load	10%	10%	15%	20%
Mobile phase	Phosphate (0.05 M, pH = 4.7)/ACN (60:40) (72:28 for TRA) (70:30 for DOX)	Phosphate/nonylamine/ACN (90:10)	Phosphate/ACN (75:25)	Phosphate (0.2 M, pH = 4.7)/ACN (80:20)
Flow-rate	2 ml/min (2.5 ml/min for TRA)	3 mL/min	2.7 mL/min	1.5 mL/min
Column Temperature	50°C (60°C for TRA, and DOX)	Amb.	43°C	50°C
Detection wavelength	254 nm (214 nm for MAP, ND-MAP, TRA, and MIA)	214 nm	254 nm	214 nm
Attenuation	0.01–0.05 AUFS	0.01–0.02 AUFS	0.001 AUFS	0.01 or 0.02 AUFS
Injection volume	50–100 μL	50 μL	350 μL	100 μL
Total analysis time	9 min (16 min for TRA)	8 min	15 min	23 min
Ref.	73,80,96,123	140	124	73

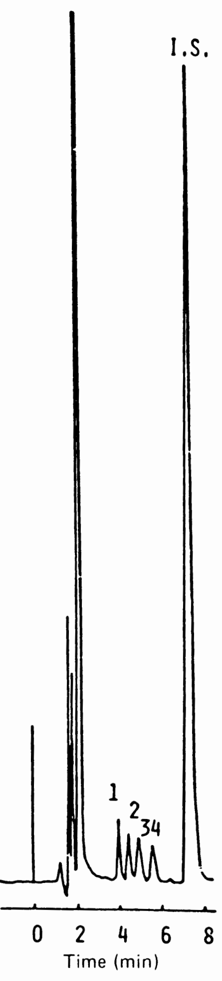


Figure 7 Chromatogram of a plasma extract containing 25 ng of each TCA per ml of plasma: 1, desipramine; 2, nortriptyline; 3, imipramine; and 4, amitriptyline. (From Ref. 123.)

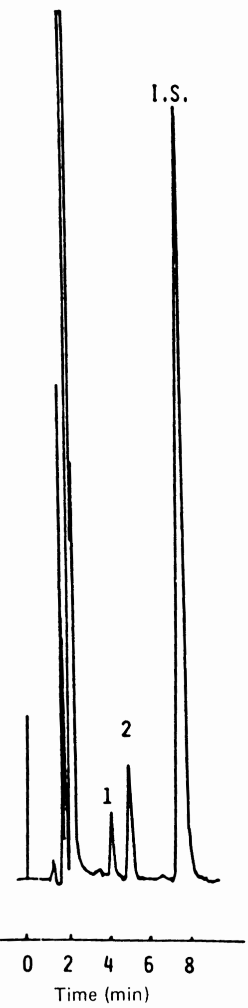


Figure 8 Chromatograms of plasma extracts from a psychiatric patient: 1, desipramine (28 ng/ml); 2, imipramine (61 ng/ml). (From Ref. 123.)

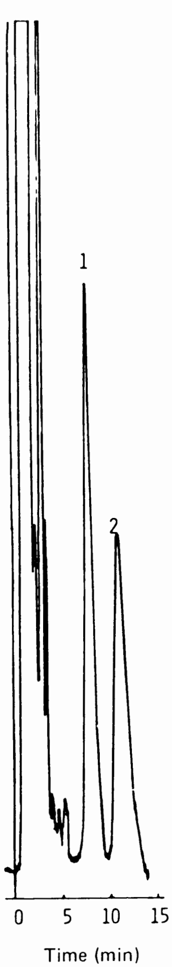


Figure 9 Chromatogram of a patient plasma extract, showing 2-OH-DES at a concentration of 51 ng/ml. Plasma sample was taken 8 hr post-ingestion. (From Ref. 124.)

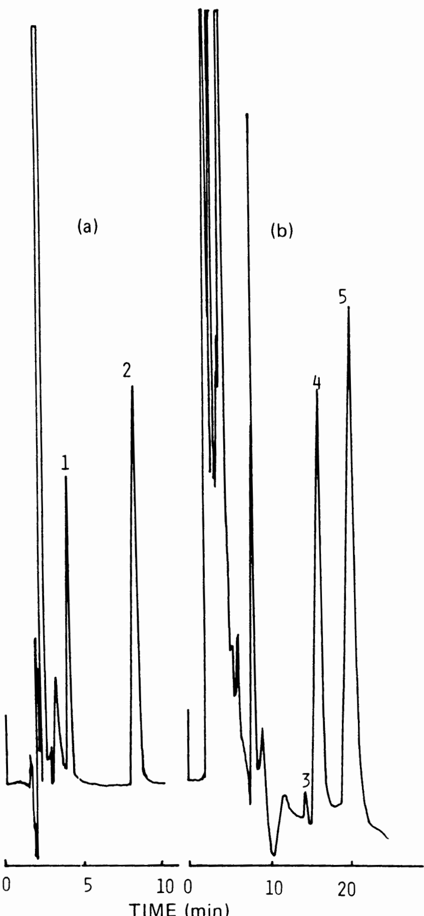


Figure 10 Chromatograms of plasma extracts from a patient receiving 150 mg AMO daily: (a) assay for AMO, (b) assay for 7- and 8-OH-AMO. Peaks: 1, AMO; 2, internal standard; 3, 7-OH-AMO; 4, 8-OH-AMO; 5, internal standard. (Reprinted with permission from *Clinical Chemistry*, Ref. 73.)

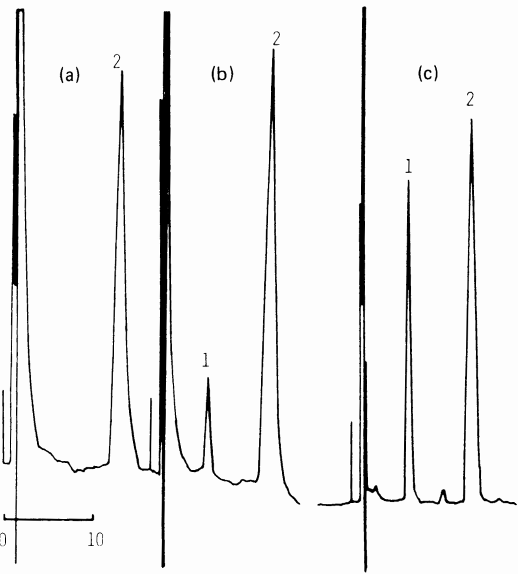


Figure 11 Chromatograms of plasma extracts: (a) with TRA-IS only; (b) with 100 μg/L of TRA (1) and TRA-IS (2); and (c) from a patient treated with TRA (150 mg daily dose), showing a TRA peak corresponding to 384 μg/L. (Reprinted with permission from *Clinical Chemistry*, Ref. 76.)

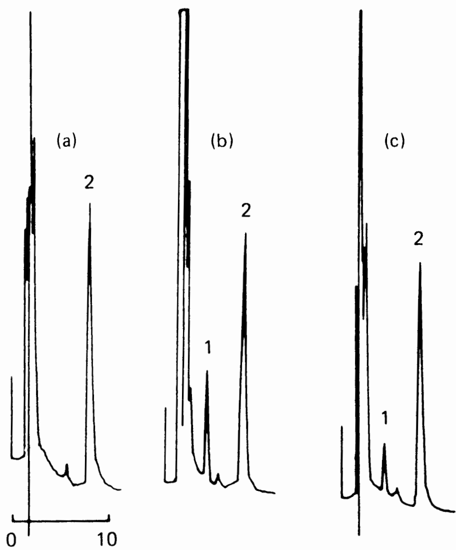


Figure 12 Chromatograms of plasma extracts (a) with clomipramine (Cl-IMI) only; (b) with 100 μg/L of MIA(1) and Cl-IMI(2); and (c) from a volunteer who ingested 40 mg of MIA, and the MIA (2) peak corresponded to 27 μg/L. (Reprinted with permission from *Clinical Chemistry*, Ref. 76.)

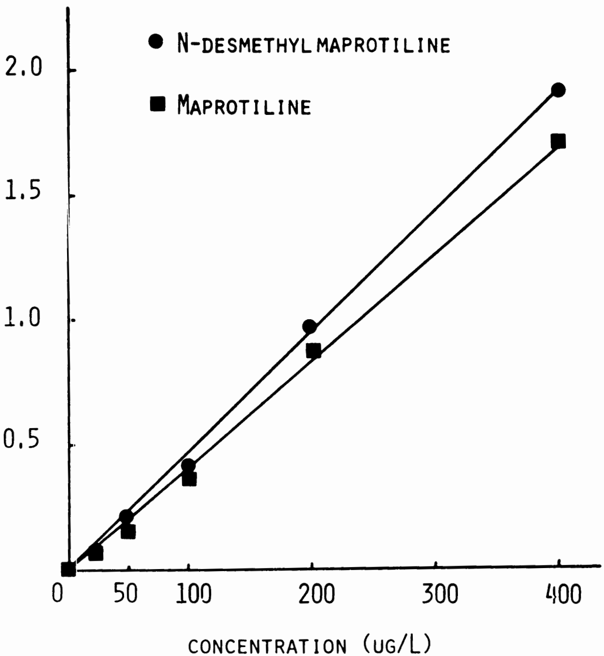


Figure 13 Calibration curves for MAP and ND-MAP: concentration vs. peak height ratios.

Sensitivity ranged from 0.5 ng for AMI, NOR, IMI, DES, to 5 ng for TRA and MIA (both from method A).

D. Conclusion

Monitoring of selected antidepressants may be readily achieved by RP-LC to answer questions related to therapeutic response, side effects, toxicity, pharmacokinetic parameters, and noncompliance. LC offers efficient and accurate determination, easily performed by the lab technologist. Thus the measurement is no longer a major laboratory problem. In order to realize the clinical efficacy of plasma levels measurements, further studies will be needed to examine their potential as a useful adjunct in the treatment of depression.

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ANTICONVULSANTS

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I. INTRODUCTION

The successful treatment of seizures with anticonvulsant drugs began in 1857 with the discovery by Charles Locock that potassium bromide reduced the frequency of seizures in some of his patients with epilepsy [1]. Fifty-five years then passed before Hauptman presented an account of his experience with phenobarbital as an anticovulsant drug [2]. Since that time, a bewildering variety of anticonvulsant drugs have been used, with additions being made to the list every year. Despite the large number of drugs available, however, six drugs are the principal agents now being used in treating patients with epilepsy: phenytoin, phenobarbital, primidone, carbamazepine, ethosuximide, and valproic acid.

The basic structure of five of these anticonvulsant drugs is a 5- or 6-membered ring derived from a condensation of urea with glycolic or malonic acid (Fig. 1). Different side-chain substitutions on the ring correlate with the clinical type of seizure which may be controlled by that compound (see Table 1). Short aliphatic substitutions, such as methyl or ethyl groups (as found in ethosuximide) are associated with effectiveness against absence seizures, whereas substitutions of larger aromatic structures, such as phenyl radicals (as found in phenobarbital and phenytoin), correlate with effectiveness against grand mal and psychomotor seizures.

(a) (b) (c)

(d) (e) (f)

Figure 1 Chemical structures of: (a) valproic acid, (b) phenobarbital, (c) phenytoin, (d) carbamazepine, (e) primidone, and (f) ethosuximide.

II. THE RATIONALE FOR THERAPEUTIC DRUG MONITORING

A. General Considerations for a Therapeutic Drug Monitoring Service

Scheme 1 outlines a typical therapeutic decision process. The scheme appears simple, yet so many pitfalls are present that the ideal therapeutic drug monitoring service is rarely found. Scheme 2 lists some of the problems which are known to occur.

The pharmacological response to a drug is a result of the interaction of the drug with tissue receptors controlling that response.

Table 1

Type of epilepsy	Drug
Tonic-clonic grand mal or partial focal ± tonic-clonic	Phenobarbital Phenytoin Primidone Carbamazepine Valproic acid
Petit mal	Ethosuximide Valproic acid

Diagnosis of disease

↓

Selection of appropriate drug regimen to establish "target" plasma drug concentration

↓

Drug administration

↓

Observation of the clinical effect of treatment

↓

Determination of drug concentration and adjustment of drug regimen to provide optimal therapeutic effect; that is, drug concentration data are used together with clinical observations to optimize therapy.

Scheme 1 A typical therapeutic decision process.

Physician elects to employ pharmacotherapy.

? Errors in diagnosis, drug choice, and dosage.

↓

Dosage prescribed by physician

? Dispensing error

? Compliance

↓

Dosage taken

? Rate and extent of absorption, distribution biotransformation, and excretion

? Liver or renal disease

↓

Serum drug concentration

? Protein concentration (albumin and others)

? Tissue uptake

↓

"Free" drug concentration

? Number of receptors

? Additional drug treatment

? Drug interaction

↓

Pharmacological effect

Scheme 2 Problems in a typical therapeutic decision process.

For most drugs the intensity and duration of the given pharmacological response is proportional to the drug concentration at the receptor site. This drug concentration depends on many factors including drug dose and the pharmacokinetic properties of the drug administered. Some of the factors affecting the pharmacokinetics of drugs include genetic differences in drug metabolism [3,4], age [5,6], drug interactions [7], diet [8,9], and many more. For many drugs it is therefore impossible to predict a serum concentration for any given mg/kg dose.

For example, greater than 10-fold differences in the steady-state serum concentrations have been found among patients treated with the same drug dosage. Figure 2 shows a frequency distribution of phenytoin serum concentrations among 200 ambulatory patients at the Clinics of the Massachusetts General Hospital, all of whom had the same daily prescribed dose of 300 mg [10]. Only 28.5% of the patients had serum concentrations between 10 and 20 mg/L, the usually accepted therapeutic range for phenytoin. Potentially subtherapeutic concentrations were present in 60% of patients while potentially toxic concentrations were found in 11.5% of patients. Clearly one cannot predict a serum concentration for phenytoin in any individual for any given dose.

For therapeutic drug monitoring to be useful in the clinical management of patients, it is necessary that the drugs of interest fulfill certain requirements. These are:

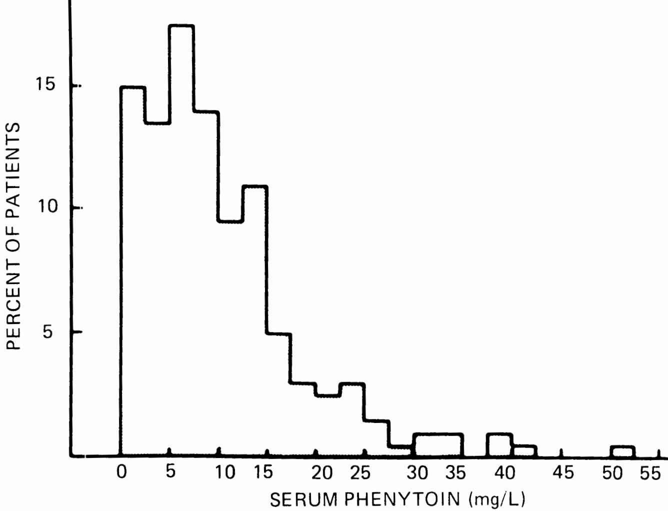

Figure 2 Frequency distribution of phenytoin serum concentrations among 200 ambulatory patients at the clinics of the Massachusetts General Hospital chronically treated with a prescribed daily oral dose of 300 mg.

1. The relationship between serum concentration and pharmacological effect must be good.
2. A reliable and rapid method for drug analysis must be available.
3. A narrow margin should exist between serum concentrations which provide therapeutic effects and those which cause toxic effects.
4. The utility of serum concentration measurement is critically dependent on the proportionate relationship between serum and tissue drug concentration.
5. There should be a poor correlation between serum concentration and drug dosage due to interindividual differences in drug absorption, metabolism, and excretion.

Most of the above criteria have been met for the anticonvulsant drugs listed in the following table. Measurement of serum drug concentrations, followed by the adjustment of dosage regimens where necessary to ensure therapeutic levels, has been shown to improve patient management [11,12].

Therapeutic drug monitoring of anticonvulsant drugs should be considered under the following conditions:

1. After therapy has been initiated but not before adequate time has elapsed for steady-state serum concentrations to be achieved.
2. When the patient responds inadequately or excessively to a standard recommended dose.
3. After new steady-state levels have been achieved following changes in drug source, dose, dosing regimen, or additions of new drugs to the dosing regimen.
4. When the patient is suspected of noncompliance.
5. As soon as possible after symptoms of toxicity occur.

The ideal specimen for analysis is serum drawn just prior to the next dose; thus, the steady-state trough concentration should be measured.

III. CLINICAL PHARMACOLOGY

The clinical pharmacology of these six drugs is summarized in Table 2, while their clinical manifestations of toxicity are presented in Table 3.

Phenytoin exhibits dose-dependent kinetics, that is, the concentration of phenytoin will increse linearly with dose until a point is reached at which the metabolizing pathways are saturated. Any slight further increase in drug dose can give rise to a large increase in serum concentrations and drug toxicity. It is important to note that while phenytoin is effective in suppressing seizures at serum concentrations of 10–20 mg/L, the drug has been known to produce an exacerbation

Table 2 Data of Interest when Monitoring Anticonvulsant Drugs

Drug	Effective plasma concentration (mg/L)	Protein bound	Elimination half-life (hrs)		Volume of distribution (L/kg)	Time to steady state (hrs) Adult/Children
			Adults	Children		
Carbamazepine	4-12	65-83	10-30	8-19	0.8-1.4	40-150
Ethosuximide	40-100	0	40-60	30-50	0.7-0.9	150-300
Phenobarbital	15-30	45-50	50-120	40-70	0.7	200-600
Phenytoin	10-20	87-93	18-30	12-22	0.5-0.8	60-150
Primidone	5-12	0-10	3-12	4-6	0.6-1.0	15-60
Valproic acid	50-100	80-95	8-15	6-15	0.15-0.40	30-75

Table 3 Clinical Manifestations of Toxicity for Anticonvulsant Drugs

Drug	Serum concentration	Clinical manifestations of toxicity
Phenobarbital[a]	40-60 mg/L	Slowness and ataxia
	60-110 mg/L	Comatose, reflexes present
	>110 mg/L	No deep tendon reflexes
Phenytoin	20-30 mg/L	Nystagmus
	30-40 mg/L	Nystagmus, ataxia
	>40 mg/L	Nystagmus, ataxia, and lethargy
	Chronic use	Gum hypertrophy, hirsutism
Primidone	>14 mg/L	Nystagmus, vertigo, ataxia, vomiting, dysarthria
Ethosuximide	>100 mg/L	Sedation, nausea, vomiting, pancytopenia
Carbamazepine	>9 mg/L	Nystagmus, drowsiness, nausea, vomiting, headache
Valproic acid	>125 mg/L	Rare but include anorexia, nausea, vomiting, and hair loss

[a]Tolerance to the sedative effect of phenobarbital is marked. Many patients may have serum concentrations as high as 75 mg/L and show no clinical signs of toxicity.

of seizures at serum concentrations greater than 40 mg/L. Phenytoin is strongly protein-bound. Any disease causing a decrease in binding can therefore be associated with a large increase in the free phenytoin concentration, leading to phenytoin toxicity. This is known to occur particularly in patients with renal failure, who may have signs of phenytoin toxicity at serum concentrations of 10-20 mg/L (usual therapeutic range). In these instances, the measurement of "free" phenytoin concentrations is recommended, the "free" concentration being most easily obtained by quantitating the concentration of phenytoin in

saliva. The dosage regimen should then be appropriately adjusted to provide a "free" concentration of 1-2 mg/L.

Phenobarbital and carbamazepine are potent inducers of the hepatic microsomal enzyme system and can markedly effect the half-life of other drugs metabolized by this route.

The protein-binding of valproic acid is variable and dependent on many factors including the concentration of valproic acid in serum. For example, at 20-60 mg/L there is about 5% free drug, at 80 mg/L there is approximately 8% free drug, and at 145 mg/L there is approximately 20% free drug [13]. Furthermore, there is competition for binding sites between valproate and phenytoin resulting in initial increased "free" concentrations of both drugs. Removal of phenytoin, carbamazepine, or phenobarbital from a drug regimen including valproic acid has been known to give rise to large increases in valproic acid serum concentrations [7]. Clearly any changes in the drug regimen should be followed shortly thereafter by drug concentration measurement and appropriate adjustment of drug regimen if required.

Primidone is converted to phenobarbital and phenylethylmalonamide, and anticonvulsant properties have been attributed to all three compounds. Therefore, phenobarbital concentrations should always be measured in patients on primidone therapy. While the primidone serum concentration is very dependent on the time of sampling relative to the time of drug ingestion (due to its short half-life), that is not the case for phenobarbital. Adjustments in the primidone dosing schedule are therefore sometimes more appropriately made on the basis of the measured phenobarbital concentration.

Finally, measurement of anticonvulsant drug concentrations is always useful in the detection of patient noncompliance with a prescribed regimen.

IV. MEASUREMENT OF ANTICONVULSANT DRUGS BY HPLC

A. Review

The ideal analytical procedure for the measurement of drug concentrations in body fluids should be:

1. Both accurate and precise, affording the reliable quantitation of the drug.
2. Technically easy, so that little effort is required in training technologists to perform the task.
3. Rapid, to minimize delay in reporting the results to the clinician. Many of the requests for drug quantitation arise as a result of problems in patient management. In our institution, results for about half the antiepileptic drugs quantitated fall

outside the therapeutic range and necessitate alterations in dose and dosing schedule. That the interval between specimen receipt and report of the result be short is therefore important.

4. Inexpensive. A key issue here is laboratory work load. It would be inappropriate to spend $30,000 for a piece of equipment (e.g., a high-performance liquid chromatograph with automated sample injector and data handling facilities) if the laboratory work load is only 10 samples per week. On the other hand, if the laboratory work load is large (greater than 40 samples per week), the purchase of such equipment would provide a cheaper service than comparable radioimmunoassay (RIA), homogeneous enzyme immunoassay (EMIT), or fluorescence polarization immunoassay (FPIA) procedures, for which reagent costs are considerable.
5. Sensitive, to allow analysis for drugs in a microscale sample and permit the simultaneous analysis of the most commonly used drugs and their pharmacologically active metabolites. Many patients with epilepsy are on multiple-drug regimens (about half at our institution).

"Nothing endures but change," and the advent and application of HPLC to therapeutic drug monitoring has been one of the most significant advances made in the clinical laboratory in recent years. Older procedures (e.g., gas chromatography) based on lengthy sample extractions and derivatization of the analyte before chromatography have been largely replaced by HPLC in today's modern laboratory.

Why this swing to HPLC? Below, I list some of the advantages of HPLC over most other analytical techniques currently in use.

1. Analyte volatility and thermal stability, so essential for gas-liquid chromatography (GLC), are not required for this type of chromatography.
2. Relatively little sample work-up is required before analysis.
3. Characteristically, methods involving HPLC require only a short analysis time.
4. There is no need for derivatization of the analyte.
5. For laboratories with a large work load, the cost per analysis is very low because reagent costs are low.
6. HPLC affords good sensitivity. The sensitivity varies with the drug, but reliable quantitation at drug concentrations as low as 1 μg/L is often achievable.
7. HPLC methods are readily automated. Routine analyses therefore require a minimum of technician time, a major factor in arriving at the low cost per analysis.
8. HPLC allows for the simultaneous analysis of most anticonvulsant drugs in a microsample.

Some limitations of HPLC in drug analysis are:

1. Spectrophotometric, fluorometric, and amperometric detectors are commonly used. Therefore, the analyte in question must either absorb light, fluoresce, or be electroactive.
2. Equipment is expensive. Investment of capital (a fully automated system currently costs about $30,000) is only warranted if the laboratory has a substantial work load (more than 40 samples per week).

Several methods that allow simultaneous analysis for phenobarbital, phenytoin, primidone, ethosuximide, and carbamazepine have been reported in the literature [15-23]. A very recent development, which has simultaneously significantly reduced column costs and increased column life, is the advent of radial compression columns (Radial Pak A, marketed by Waters Associates, Inc., Milford, Massachusetts). Using these columns, Soldin [22,23] has recently described a procedure for the simultaneous analysis of five anticonvulsant drugs and their active metabolites.

Although HPLC analysis of valproic acid has been described [24, 25], these procedures require either postcolumn reactions [24] or precolumn derivatization [25]. In my opinion, the method of choice for the analysis of valproic acid is still GLC.

B. Analysis

1. *Recommended Method: HPLC [18-23]*

Principle: The five anticonvulsant drugs, primidone, phenobarbital, ethosuximide, carbamazepine, and phenytoin; the primidone metabolite, phenylethylmalonamide; and the carbamazepine metabolite, carbamazepine-10,11-epoxide are separated by reversed-phase liquid chromatography and quantitated by measuring peak areas relative to the peak area of the internal standard, dihydrocarbamazepine. The compounds are identified by their retention times and also by their absorbance ratio at 200 and 254 nm. Each drug has a known absorbance ratio. Any skewing of the ratio would alert the operator to the presence of an interfering compound.

Reagents and Materials: We have employed a high-performance liquid chromatograph, model ALC/GPC 204/6000A, with an autosampler (WISP, model 710A), a radial compression system (RC-100) and a 10 × 0.8 cm radial-PAK A column, all purchased from Waters Associates, Inc. (Milford, Massachusetts). Detectors used were the Waters model 440 with 254-nm kit and the model LC55 variable-wavelength spectrophotometer (Perkin-Elmer Corporation, Norwalk, Connecticut). The recorder was a Honeywell Electronic, model 196 (Honeywell, Inc., Fort Washington, Pennsylvania). A Sigma 10 data handling system (Perkin-Elmer) was employed to receive and compute the spectrophotometer signals.

We obtained acetonitrile, methanol, and dimethylsulfoxide from Burdick and Jackson, and standards from Applied Science Laboratories (phenobarbital), Parke-Davis (phenytoin and ethosuximide), Ayerst Laboratories (primidone), Aldrich Chemical Company (10,11-dihydrocarbamazepine), and Geigy (carbamazepine).

Procedure: Acetonitrile (25 μL) containing approximately 200 mg/L 10,11-dihydrocarbamazepine is added to 25 μL or serum or plasma. The sample is vortexed for 30 sec, centrifuged, and then 4 μL is injected into the liquid chromatograph. The chromatograph conditions are shown in Table 4. The chromatogram obtained using this procedure is shown in Fig. 3.

Comments: We are unaware of interferences in the method described and have performed over 20,000 analyses with this system. Each morning the system is calibrated using Ortho Bi-level Anticonvulsant/Antiasthmatic controls (product code 9086, Ortho Diagnostics, Inc., Raritan, New Jersey). The between-day precision is shown in Table 5.

The procedure described above provides for the simultaneous analysis of five anticonvulsant drugs; the primidone metabolite, phenylethylmalonamide; and the carbamazepine metabolite, carbamazepine-10, 11-epoxide. Although HPLC methods have been reported for the measurement of valproic acid in serum or plasma, the analysis of valproic acid is most readily accomplished by GLC [26-29] or EMIT procedures.

EMIT or FPIA procedures provide an acceptable alternative to the HPLC method described above. For a laboratory with a large work load they are, however, considerably more costly although they have the advantage of great simplicity. It should be noted that in a pediatric institution the laboratory will receive many samples for analysis

Table 4 Chromatographic Conditions for Anticonvulsant Drug Assays by HPLC

Temperature	room
Mobile phase	
Acetonitrile/10 mM phosphate buffer	37/63
pH	7.3
Elution rate, ml/min	2.0
Absorbance	200/254
Sample injected	4 μl
Analysis time	10 min

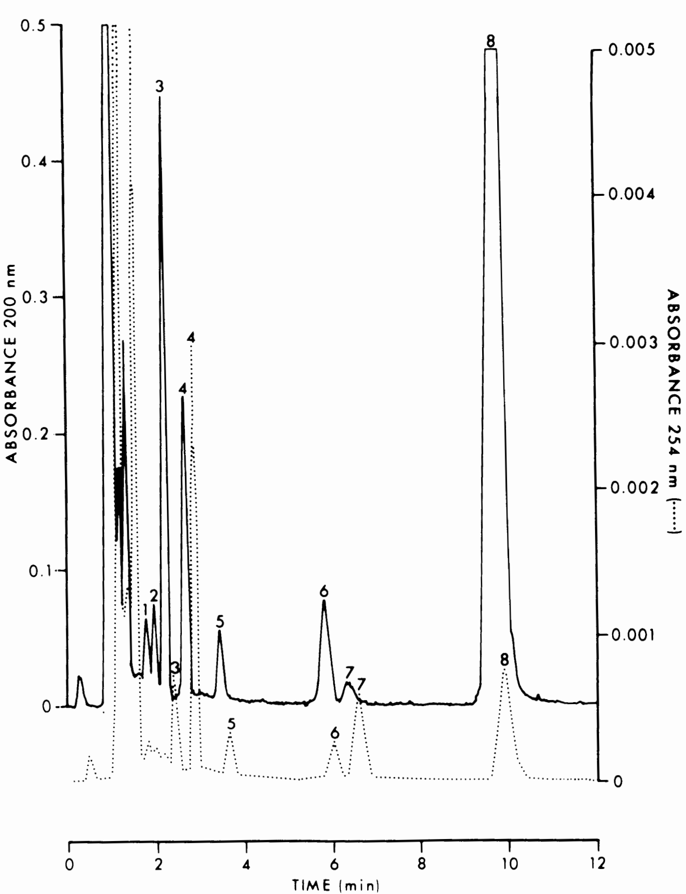

Figure 3 Chromatogram obtained for a plasma specimen containing therapeutic concentrations of primidone (2), ethosuximide (3), phenobarbital (4), phenytoin (6), and carbamazepine (7). Peaks 1, 5, and 8 represent phenylethylmalonamide, carbamazepine-10,11-epoxide, and the internal standard, dihydrocarbamazepine, respectively (—— 200 nm), (---- 254 nm).

Table 5 Between-day Precision

Drug	Concentration (mg/L)	CV%
Primidone	13	5.7
Ethosuximide	110	1.7
Phenobarbital	40	3.1
Phenytoin	20	6.8
Carbamazepine	8	7.3

which are visibly hemolyzed, and hemolysis is known to interfere with the precision of EMIT chemistries.

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13

ANTIHYPERTENSIVES

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I. INTRODUCTION

Hypertension is one of the most prevalent chronic disorders in the United States. It is estimated that 12–35 million persons in this country may have high blood pressure, and at least one-third of these are not aware of their disease. About 5 million hypertensives take blood pressure-lowering medications. The risk of cardiovascular disease is considered higher in persons with elevated blood pressure (even moderately) than in the normotensive population [1,2]. Although antihypertensive drugs have contributed significantly to the decline in cardiovascular disease-related morbidity and mortality, the control of hypertension and its associated risks can be significantly decreased if the disease is detected early and appropriate drug therapy is instituted.

From time to time, a large number of natural and synthetic compounds have been screened for their ability to lower blood pressure; some of these have been tested more thoroughly. In a recent survey (1979–1982) of world literature on antihypertensive drugs, the data were compiled on more than 500 agents [3]. The number of clinically effective antihypertensive agents is ever increasing. However, this review will cover the high-performance liquid chromatographic (HPLC)* methods of analysis of important drugs, primarily those which are approved for clinical use in the United States. The mode of action and selected pharmacokinetic parameters of these drugs in man are also presented.

Antihypertensive drugs have been broadly classified, based on mechanisms and/or sites of blood pressure-lowering action, into the following categories: (a) centrally acting drugs; (b) drugs interacting at α-adrenoceptor sites; (c) β-andrenoceptor antagonists; (d) drugs interacting with the autonomic nervous system by mechanisms other than (b) or (c); (e) inhibitors of the renin-angiotensin system; (f) diuretics; (g) vasodilator antihypertensives; and (h) other drugs (having miscellaneous mechanisms and sites of action).

Plasma level monitoring of antihypertensive agents will be especially beneficial in cases of unusual drug toxicity, apparent drug resistance, and in patients with kidney or liver disorders with a potential to develop adverse drug reactions.

II. DRUGS WITH CENTRAL MODES OF ACTION

A. Reserpine

Reserpine acts by a complex mechanism which is not fully understood. Part of the antihypertensive effect of reserpine is via depletion of endogenous catecholamine stores of the postganglionic adrenergic neurons and the adrenal medulla. The central effect may be due to mobilization of norepinephrine which, in turn, stimulates α-adrenoceptors in the CNS. Blood pressure is decreased as a result of a reduction in peripheral vascular resistance by the peripheral and central effects of reserpine.

Reserpine is extensively metabolized. Its oral absorption is poor and the therapeutic doses are low. Therefore, only low levels of the drug are achieved in plasma of patients receiving the drug. The presently available HPLC methods are not sensitive enough to be of value in pharmacokinetic studies of reserpine. However, these methods are valuable in quantitating the drug and the impurities in dosage formulations.

Commercially available brands of reserpine tablets contain 3,4-dehydroreserpine, 3-isoreserpine [4] and 3,4,5,6-tetrahydroreserpine. Prior to the advent of HPLC, analysis of reserpine formulations was based on either direct UV determination or oxidation of reserpine

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*Abbreviations used: CNA = central nervous system. CV = coefficient of variation, EC = electrochemical, GC = gas chromatography, GC-ECD = gas chromatography-electron capture detector, GC-MS = gas chromatography-mass spectrometry, RIA = radioimmunoassay, TLC = thin-layer chromatography; $t_{\frac{1}{2}}$ = elimination half-life, $t_{\frac{1}{2}}\alpha$, $t_{\frac{1}{2}}\beta$, $t_{\frac{1}{2}}\gamma$ = the first, second, and third component $t_{\frac{1}{2}}$s of a triphasic plasma level-time curve, V_d = apparent volume of distribution; AcOH = acetic acid, EDTA = ethylenediaminetetraacetate, Et_2O = diethyl ether, EtOAc = ethyl acetate, MeOH = methanol; i.v. = intravenous.

followed by UV or fluorometric estimation [5]. Using reversed-phase HPLC, reserpine was analyzed in multicomponent dosage forms [6], but the procedure was found to be less satisfactory (long retention times, wide peaks, and questionable stability of reserpine at pH 8 of the solvent system used). A normal-phase HPLC was also employed [7] to quantitate reserpine in dosage forms (also containing hydrochlorothiazide), but this procedure did not separate and quantify the degradation products and impurities found in reserpine tablets.

An improved HPLC method using reversed-phase RP-8 column (25 cm × 0.46 cm, octylsilane on 10-μm LiChrosorb), which separates reserpine from its degradation products, was used to quantitate reserpine (UV, 254 nm) and degradation products (fluorescence detection, excitation at 330 nm, emission at 470 nm). Elution was carried out with a 50:50 mixture of MeOH:0.05 M NaH_2PO_4, pH 4.5, pumped at a flowrate of 2 mL/min. Extracts of tablets in ethyl acetate with the internal standard (propiophenone) were injected directly on the column. The retention times (and detection modes) were: internal standard (UV) 6.6 min; resperine (UV) 15 min; 3,4,5,6-tetrahydroreserpine (fluorescence) 18 min; 3,4-dehydroreserpine (fluorescence) 20 min; 3-isoreserpine (UV) 30 min. Detector response was linear for 0.25-4 μg in 10 μL of sample injected. Using this method, it was shown that tablets contained reserpine within the official limits even 10 years following manufacture [5].

B. α-Methyldopa (α-MD)

The antihypertensive effect of α-MD is mediated by its active metabolites, α-methyldopamine and α-methylnorepinephrine, in the CNS. Acute or chronic administration of α-MD decreases systemic vascular resistance via stimulation of central α-adrenergic receptors by the metabolites. This results in a reduction of blood pressure.

After oral administration, α-MD is rapidly absorbed, but the bioavailability varies widely (8-62%) in different individuals. Plasma elimination $t_{\frac{1}{2}}$ is less than 2 hr, and about 40% of the drug is sulfated in the first pass through the liver.

Jenner and Brown [8] employed electrochemical detection with HPLC to develop a sensitive and specific method for measurement of α-MD in plasma. Plasma (1-4 mL) mixed with the internal standard (dihydroxybenzylamine) was applied to an alumina column at pH 8.5. The column was eluted with 200 μL of 0.07 M H_3PO_4. The eluate (100 μL) was buffered with 30 μL of 0.27 M tripotassium citrate (in mobile phase) and injected on an Ultrasphere octyl reversed-phase column. The mobile phase consisted of a mixture of 70 mM KH_2PO_4, 1 mM EDTA, 6 mM octanesulfonate, and 14% MeOH, adjusted to pH 6.4. The detector was a glassy carbon electrode at an operating potential of +0.5 V. This procedure, which also quantitated epinephrine, norepinephrine,

and α-methylnorepinephrine, was relatively free from interference and considered simpler and less expensive than other procedures such as radioenzymatic assay.

The method of Cooper et al. [9], which measures α-MD and its metabolites, also appears to be simple and rapid: serum (0.1-1.0 mL) was deproteinated by the addition of 50 μL of 70% $HClO_4$, followed by mixing, chilling, and centrifugation. The supernatant fraction was filtered through an Amicon Centriflow membrane cone, and 10-μL aliquots of the filtrate were injected on a cation-exchange column (50 cm × 0.2 cm, Vydac SCX cation exchange resin, 30-44 μm). The column was eluted with 20 mM $NH_4H_2PO_4$ (pH 2.55) containing 0.1 mM EDTA, pumped at a flow-rate of 0.4 mL/min. Electrochemical detection (+0.54 V against Ag/AgCl) increased the sensitivity of the method to 50 ng/mL. Standard curves were linear over the range of 0.05-5.0 μg/mL, and recovery of α-MD from serum was 89 ± 4%.

Ong et al. [10] reported that the above procedure did not give reproducible results because of the instability of α-MD and its conjugates. These investigators were able to improve the procedure by stabilizing the drug in blood collection tubes (with EDTA and sodium metabisulfite) and reducing the time of contact of acid precipitant and supernatant to a minimum. After extraction, α-MD was analyzed by reversed-phase chromatogrpahy with electrochemical detection. Recently, Gelber and Neumeyer [11] have determined the enantiomeric purity of α-MD by using chiral mobile-phase HPLC. Several other HPLC methods for the measurement of α-MD have been described in the literature [12-15].

Measurement of plasma levels of α-MD, a drug which exhibits large intersubject and intrasubject variation in bioavailability, may be valuable in cases where poor absorption of the drug is suspected or in patients who are likely to experience adverse effects from this drug.

C. Clonidine

Clonidine, a potent antihypertensive agent, lowers blood pressure and heart rate by stimulating postsynaptic α_2-adrenergic receptors in the central nervous system.

Clonidine is rapidly absorbed when given orally; its systematic bioavailability is about 75% of the dose. After an oral dose, plasma $t_{\frac{1}{2}}$ of clonidine is in the range of 6-23 hr (6-11 hr when given i.m.), and its hypotensive effect lasts for 6-10 hr. About 40-50% of the dose is metabolized; the metabolites are inactive. The effective dose of clonidine is fairly low (0.3 mg) and its therapeutic window is in the range of 0.2-2.2 ng/mL in plasma. Plasma levels of the drug have been measured by GC, GC-MS [16-18], and RIA [19,20]. An HPLC method for the measurement of clonidine in tablets has recently been described [21]. The drug was extracted with $MeOH-H_2O$, and the filtered solution was injected on a 5-6 μm trimethylsilyl bonded spherical silica

column (25 cm × 0.4 cm). The column was eluted with 85% MeOH in pH 7.9 phosphate buffer pumped at 1 mL/min. The detector was set at 254 nm. Clonidine and the internal standard (chlorthalidone) eluted at 3.5 min and 7.5 min, respectively.

D. Guanabenz

A new centrally acting drug, guanabenz, resembles clonidine closely and produces its hypotensive effect by causing a reduction of central sympathetic outflow by stimulation of central α-adrenoceptors.

After oral ingestion, about 75% of the drug is absorbed. Maximum plasma levels are reached in 2-5 hr; $t_{\frac{1}{2}}$ of the drug is about 6 hr. Guanabenz is extensively metabolized; *p*-hydroxyguanabenz is the major metabolite appearing in the urine. Only GC and isotopic methods are available for the measurement of guanabenz in plasma [22]. The stability of the drug in dosage formulations has been measured by HPLC [23].

III. DRUGS ACTING AT THE α-ADRENOCEPTOR SITES

A. Prazosin

Prazosin, a quinazoline derivative, appears to act mainly by postsynaptic α_1-adrenoceptor blockade, but the mechanism of action in man has not been fully characterized [24,25]. Prazosin, given orally, is rapidly absorbed, with peak plasma levels achieved in about 1 hr. The drug disappears from plasma with a $t_{\frac{1}{2}}$ of 2-3 hr. The first-pass metabolism accounts for about 14% of the oral dose of prazosin; bioavailability varies between 55 and 82% of the dose [24-27]. Prazosin is extensively metabolised in man [24]. Several metabolites (6-O-desmethylprazosin, 7-O-desmethylprazosin, and 2,4-diamino-6,7-dimethoxyquinazoline) have been shown to be active in the dog and rat [28,29]. These active metabolites may account for the longer dynamic (antihypertensive and adrenoceptor blocking) effects of the drug (up to 7 hr) than would be expected from its short $t_{\frac{1}{2}}$ [27].

Several sensitive and specific HPLC methods have been developed to analyze prazosin and certain metabolites in plasma [30-36]. Yee et al. [32] developed a simple, rapid, and sensitive method for measurement of prazosin in whole blood or plasma. Blood or plasma (0.1-1.0 mL) was spiked with 0.1 mL of a solution of the internal standard [2-methallyl ester of 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid] and alkalinized with 0.2 mL of 2 N NaOH. The mixture was extracted with Et_2O (5 mL). The organic phase was reextracted with 0.1 N H_2SO_4 (20 μL). The aqueous phase was injected on a C_{18} Micro-Pak MCH-10 reversed-phase column (25 cm × 0.2 cm). The mobile phase consisted of a 49:51 v/v mixture of MeOH:aqueous 0.01 M pentane sodium sulfate adjusted to pH 3.4 (AcOH) pumped

at a flow-rate of 0.67 mL/min. A fluorescence detector was used (excitation at 253 nm, emission at 390 nm) to monitor the eluate. The method is sensitive and reproducible with a detection limit of 0.2 ng/mL prazosin in whole blood and 0.5 ng/mL in plasma. Many of the other antihypertensive drugs either do not interfere or the method can be modified to overcome the interference.

Grahnén et al. [30] modified the extraction procedure of Twomey and Hobbs [31] for the measurement of prazosin. Plasma (1 mL) was alkalinized with 1 M NaOH (0.5 mL) and then washed with 4 mL of *n*-octanol. The aqueous layer was mixed with 0.2 mL of CH_3CN and saturated with solid Na_2CO_3. After mixing and centrifugation, aliquots of the CH_3CN phase were injected on the reversed-phase column. The sensitivity of the method was 0.2 ng/mL prazosin in plasma.

A correlation between serum levels of prazosin (measured by the HPLC method) and decrease in blood pressure in the β-elimination phase was shown when the drug was given intravenously, but not when given orally [37,38].

Using the procedure of Yee et al. [32], Rubin and co-workers [39] demonstrated that age influences the bioavailability (decrease from 68% to 48%), $t_{\frac{1}{2}}$ (increase from 2.0 hr to 3.2 hr) and V_d at steady state (increase from 0.63 L/kg to 0.89 L/kg), but not the clearance (3.94 vs. 3.53 L/min/kg) of prazosin in healthy volunteers given the drug orally or i.v. The disposition of prazosin is also altered in congestive heart failure [40].

HPLC methods have been described for the measurement of the antihypertensive agents indoramine [41] and phentolamine [42,43] which act via α-adrenoceptor blockade.

IV. β-ADRENORECEPTOR ANTAGONISTS

Blockade of β-adrenergic receptors was first noted in 1964 by Pritchard [44,45] to produce an antihypertensive effect in man. Propranolol, the first beta blocker used in the United States for the treatment of hypertension, has been the most popular drug in this category. Several beta blockers have recently been introduced as antihypertensive agents.

Beta blockers competitively inhibit catecholamine binding to β-adrenoceptors on cell membranes. The mechanism of the antihypertensive action of beta blockers is not well understood. Some of the mechanisms proposed (see Ref. 46) include: (a) inhibition of renal renin release with subsequent inhibition of angiotensin II-induced vasoconstriction in the resistance vessels, (b) decrease in cardiac output, (c) changes in baroreceptor sensitivity, (d) direct, centrally mediated decrease of sympathetic outflow, (e) action via metabolites, and (f) peripherally mediated input damping. It is conceivable that several of these mechanisms may be operational in the antihypertensive effect of a beta-blocking agent in a particular patient.

β-Adrenoceptor blocking agents may be classified as selective or nonselective. The cardioselective drugs completely inhibit β-receptors in the heart, with minimal to no influence on peripheral and bronchial β-adrenoceptors. The nonselective drugs block β-receptors in the heart and the periphery. Some drugs have partial agonist effect (intrinsic sympathomimetic activity). Others have quinidine-like action (membrane-stabilizing property). Some drugs have both α- and β-adrenoceptor blocking activity [47-49].

All β-adrenergic blocking drugs will lower arterial pressure in 30-60% of all hypertensive patients. They have relatively flat dose-response relationships. To enhance their efficacy, beta blockers have been used successfully in combination with vasodilators [49]. Recently, agents have also been developed which possess both beta blocking and vasodilator properties within a single molecule, e.g., prizidilol [47,48,50].

The β-adrenolytic activity of the racemic beta blockers resides almost entirely in the levorotatory isomer (most methods for the measurement of beta blockers do not discriminate between the two isomers). Excellent correlations have been obtained between beta-blocking effect and the log of the plasma levels of most beta blockers (see Ref. 46), both after acute and chronic administration of these drugs.

Clinical studies have demonstrated large interindividual variations in the effective dosages of beta blockers, partly due to differences in metabolism (propranolol, acebutolol) and intestinal reabsorption after biliary excretion. There is a great variation in the plasma levels that produce the same therapeutic effect in different patients. For example, there was a fivefold range in the daily doses of propranolol (400-2000 mg) required by end-stage renal disease patients to adequately control their blood pressure; plasma levels in these patients ranged from 125 to 2000 ng/mL [51,52]. Differences in levels of free drug and of active metabolites as well as in receptor sensitivity may account for this large variation in the effective levels of propranolol in plasma.

Most beta blockers are absorbed fairly rapidly after oral administration; maximum plasma levels are achieved in 1-2 hr (2-4 hr for acebutolol and atenolol). At therapeutic doses, the bioavailability may vary from less than 20% for propranolol to more than 90% for pindolol and sotalol. A high first-pass elimination is observed for alprenolol, labetalol, metoprolol, oxprenolol, and propranolol. The variable bioavailability and high first-pass effect cause considerable interindividual variation (4 to 20-fold) in plasma levels after oral administration of a given dose of beta blockers [47,48,52,53].

Plasma $t_{\frac{1}{2}}$ for most of these agents is in the range of 2-4 hr, except for atenolol (5-9 hr), nadolol (17-24 hr), and sotalol (5-18 hr). Despite the relatively short $t_{\frac{1}{2}}$ of beta blockers, most of these drugs can be given in one daily dose to achieve consistent blood pressure control [47-49]. Most of the beta blockers are eliminated primarily by metab-

olism (except atenolol, nadolol, and sotalol), and in some cases the metabolites are active (alprenolol, acebutalol, and propranolol).

Many of the beta-blocking agents have been quantitated by nonspecific fluorescence measurement procedures. The majority of specific methods for measurements of beta blockers involve GLC techniques which require derivatization prior to injection on the column (see Ref. 55). HPLC is beginning to replace GLC to provide a simpler and more specific method for quantitation of these drugs and their metabolites [54]. The sensitivity and specificity of the HPLC procedures have been further enhanced by the use of fluorescence or electrochemical detectors (for certain drugs). Lefebvre et al. [55] developed optimum conditions for measurement of nine beta blockers in plasma and urine by HPLC. Patel et al. [56] have described an HPLC procedure which can be used to determine 11 beta blockers in dosage formulations.

A. Propranolol

Propranolol is a nonselective beta blocker. After oral absorption, the drug is rapidly and fairly completely (>90%) absorbed. Because of high first-pass effect, the bioavailability of propranolol is low (about 20-30%) and is dose dependent. Plasma levels achieved at a dose level may vary 20-fold in different individuals. The plasma $t_{\frac{1}{2}}$ is in the range of 2-4 hr. Propranolol is extensively metabolized (>95%). Of the known metabolites of propranolol, the 4-hydroxy metabolite is active.

Many HPLC procedures have been described for the quantitation of propranolol and its metabolites [54-81]. In a typical method [74], plasma (1 mL) is spiked with a suitable amount of an internal standard (*n*-butyrylphenoxy analogue of acebutolol, MB 19421) and then made alkaline with 0.5 mL of 10 N NaOH. The alkalinized plasma is shaken with 6 mL of 4:1 v/v $Et_2O:CHCl_3$ and then centrifuged. The organic layer is removed, evaporated to dryness, and the residue reconstituted in 100 μl of the mobile phase (0.01 M PO_4 buffer, pH 5.0:CH_3OH:CH_3CN, 6:3:1 v/v). Suitable aliquots of this solution are injected on a 15 cm × 0.46 cm Spherisorb nitrile-bonded silica (5 μm) for the separation of propranolol and the internal standard. Native fluorescence is used for detection (excitation at 254 nm and emission at 340 nm). The coefficient of variation for normal human plasma to which propranolol was added = 3.1% at 20 ng/mL and 1.7% at 100 ng/mL; limit of detection = 2 ng/mL.

Jackman et al. [75] extracted propranolol from plasma (2 μL) into 1% methanolic heptane and reextracted into 0.5 mL of 0.1 N HCl. Aliquots of the acidic solution were injected on a Zorbax C_8 reversed-phase column (25 cm × 0.4 cm). The mobile phase (CH_3CN:0.01 M $HClO_4$, 9:1 v/v) was pumped at a flow-rate of 2 mL/min. A fluorescence detector (excitation 220 nm, emission 320-400 nm) monitored the eluate.

Using an HPLC method, Reimann et al. [82,83], showed that cimetidine significantly increased plasma steady-state levels of propranolol, and this may cause enhancement of the effect of a given dose of the beta blocker. These authors used *N*-ethylpropranolol as the internal standard and extracted propranolol and the internal standard from the alkalinized plasma into heptane-isoamyl alcohol. The organic phase was extracted into acid; the aqueous solution was alkalinized and then reextracted with the organic phase. After evaporation of the solvent, the residue was reconstituted in the mobile phase and injected on the column. The limit of sensitivity of this assay was about 0.5 ng/mL propranolol in plasma.

Pritchard et al. [61,62] have described an HPLC method for the measurement of propranolol and six of its metabolites (including 4-hydroxy compound) in urine. These authors used a 30 cm × 0.4 cm C_{18} μBondapak column and fluorescence detection. Albani et al. [78] developed a simple and rapid method for measurement of propranolol and 4-hydroxypropranolol in human plasma. They used a LiChrosorb CN column and fluorescence detection technique (excitation at 285 nm, emission at 405 nm).

Schneck et al. [84], using an HPLC method [64], separated and quantitated four metabolites of propranolol in plasma and urine of young healthy adults given increasing single oral doses of the drug (20-360 mg). As the dose of propranolol was increased, its intrinsic clearance decreased and $t_{\frac{1}{2}}$ increased, probably as a result of reduction in hepatic blood flow secondary to enhanced β-adrenergic blockade at higher plasma propranolol levels. These authors also showed that the acidic metabolites of propranolol accumulate in patients with severe impairment of renal function. Hitzenberger et al. [85] observed that there we an age-related increase in peak plasma levels and a decrease in the elimination rate in patients receiving propranolol.

Thompson et al. [86] were able to separate and quantitate the *d*- and *l*-isomers of propranolol by reacting the racemic drug with *R*-(+)-1-methylbenzyl isocyanate. The diastereoisomers were separated on a Spherisorb 5-μm C_{18} column. The elution solvent was MeOH: H_2O (70:30, v/v); a fluorescence detector was used to monitor the eluate (excitation at 220 nm, emission at 340 nm). The *d*- and *l*-isomers had retention times of 9.6 and 10.3 min, respectively. The minimum detectable amount was 0.2 ng of each isomer of propranolol. Using this procedure it was found that the *l*-enantiomer (which is the active form) disappears more rapidly from the body than the *d*-isomer.

Von Bahr et al. [87] showed that plasma levels of *l*-propranolol were consistently higher than those of *d*-propranolol after oral administration but not after i.v. injection of the racemic drug. Plasma $t_{\frac{1}{2}}$ was not different for the two isomers.

The *d*- and *l*-isomers of propranolol were also separated as diastereoisomers by reversed-phase ion-pair chromatography [88-90].

B. Metoprolol

Metoprolol is a cardioselective β-adrenoceptor blocker. After oral administration it is almost completely (>95%) absorbed; the bioavailability is ~50% of the dose. Peak plasma levels are achieved in about 2 hr, and plasma $t_{\frac{1}{2}}$ of the drug is in the range of 2-5 hr. Metoprolol is extensively metabolized. One of the major metabolites, α-hydroxymetoprolol, is active and may be clinically important if accumulated. A genetic polymorphism in the metabolism of metoprolol has been reported [91-93], but some investigators did not observe this phenomenon [94].

Several HPLC methods have been described for the quantitation of metoprolol [55,56,76,95,96]. Lefebvre et al. [55] extracted metoprolol from plasma (1 mL) after alkalinization (100 μL of 1 N NaOH) with 7 mL of $CHCl_3$:*n*-pentanol, 60:20 v/v. The organic phase was extracted with 0.1 N H_2SO_4 (0.3 mL). Aliquots of the aqueous phase were injected on a μBondapak C_{18} column (30 cm × 0.4 cm). Separation was achieved by using a mobile phase consisting of MeOH:AcOH:H_2O (50:1:49, v/v/v) pumped at a flow-rate of 1.3 mL/min. A fluorescence detector (Schoeffel, with no filters, excitation at 222 nm) was employed; the internal standard was dibutolol (metabolite of acebutolol). The detection limit was 10 ng/mL. The retention times for metoprolol and the internal standard were 4.8 and 8.3 min, respectively. Using the above procedure, Lennard et al. [91,92] showed that genetically poor hydroxylators of debrisoquin also had defective metabolism of metoprolol. Poor hydroxylators also had longer plasma $t_{\frac{1}{2}}$ of the *R*-isomer than for the *S*-isomer.

Winkler et al. [76] have described a simple and rapid HPLC method for quantitative analysis of metoprolol in plasma and tissues. Plasma (1 mL) was alkalinized (0.5 mL, 2 N NaOH) and extracted with 3 mL of 1-butanol:*n*-heptane (10:90, v/v). The organic layer was extracted with 0.1 N H_2SO_4 (100 μL), and aliquots of the acidic layer were injected on a LiChrosorb C_{18} (10 μm) column (25 cm × 0.46 cm). The mobile phase consisted of a 60:40 v/v mixture of MeOH:H_2O (containing 0.0025 M octyl sodium sulfate-1.3% H_3PO_4) pumped at a flow-rate of 2 mL/min. Detection was achieved by fluorescence measurement (excitation at 280 nm, emission at 300 nm). Metoprolol was extracted from tissues by homogenization in 0.4 N $HClO_4$ followed by centrifugation. The supernatant fraction was alkalinized and then treated as described for plasma. Recovery of drug from plasma and tissues was about 50%. The detection limit was 2 ng/mL for plasma and 5 ng/g for tissue.

The *R*- and *S*-isomers of metoprolol have been separated (as diastereoisomers) by ion-pair reversed-phase chromatography [90,97].

C. Atenolol

Atenolol is a cardioselective beta blocker. After oral administration, 30-60% of the dose is absorbed. It has a plasma $t_{\frac{1}{2}}$ of 5-9 hr. Only

10-15% of the dose is metabolized and the remainder is excreted unchanged in the urine. The disposition of atenolol is not significantly altered with age [98].

Atenolol was measured in plasma (1 mL, alkalinized with 0.1 mL of 0.1 N NaOH) after extraction with CH_2Cl_2:*n*-pentanol, 80:20, v/v (7 mL) and reextraction into 0.3 mL 0.1 N H_2SO_4. Aliquots of the aqueous phase were injected on a μBondapak C_{18} column (30 cm × 0.4 cm) and eluted with MeOH:AcOH:H_2O, 29:1:71 v/v pumped at 1.3 mL/min. A Schoeffel fluorescence detector (without a filter) and excitation wavelength of 222 nm was used to monitor the effluent. The retention times of atenolol and procainamide (used as internal standard) were 6.4 and 8.4, respectively. The detection limit was 10 ng/mL atenolol in plasma [99]. A similar, but more sensitive procedure for extraction and quantitation of atenolol is described by Winkler et al. [76].

Holt et al. [100] have described a rapid HPLC method for the measurement of atenolol. The sample or standard solution (0.25 mL) was mixed with sodium chloride (50 mg), the internal standard (50 μL of a 0.2 mg/L solution of benzimidazole), 10 M NaOH (50 μL), and heptafluorobutanol (0.2 mL of a 10% v/v solution in methyl *t*-butyl ether). After mixing and centrifugation, 100-μL portions of the organic layer were analyzed on a 5-μm Spherisorb 5 silica column (12.5 cm × 0.5 cm) using MeOH containing 230 mg/L camphor sulfonic acid as the eluting solvent. The fluorescence of the effluent was monitored using 195 nm excitation wavelength). Atenolol and the internal standard eluted at 3.7 and 4.9 min, respectively. Several other HPLC methods for the measurement of atenolol have been published [56,101,102].

D. Labetalol

The drug has both α- and β-adrenoceptor blocking as well as direct vasodilating properties [103]. After oral administration, the drug undergoes significant first-pass effect, and there is a large interindividual variation in plasma levels and oral bioavailability (9-68%). The plasma $t_{\frac{1}{2}}$ is in the range of 1.7–7.0 hr [104]. Labetalol is extensively metabolized; less than 5% of the dose is excreted in urine as unchanged drug. The major metabolites (*N*-glucuronide, *O*-phenylglucuronide, and alcohol glucuronide) are pharmacologically inactive [104]. Plasma $t_{\frac{1}{2}}$ and bioavailability of labetalol increase with age [105].

Labetalol was measured in plasma by the following procedure [106]. Plasma (1 mL) to which 20 μL of propranolol (5–10 mg/L, as internal standard) had been added was adjusted to pH 8.5 (with Britton-Robinson buffer, consisting of 0.2 M each of acetic, boric, and orthophosphoric acids adjusted to pH 9.2 with 1 N NaOH), and then extracted with 5 mL of $CHCl_3$ (shaken for 5 min and centrifuged for 10 min). The organic layer was extracted with 50 μL of 0.1% H_3PO_4. The aqueous layer was washed with 2 mL and then with 1 mL of hexane, and 15-μL aliquots of the aqueous phase were injected on a μBondapak C_{18}

column. Elution was carried out with a solvent consisting of 35% CH_3CN in 0.1% aqueous H_3PO_4, flowing at a rate of 1 mL/min. The detector was set at 205 nm. The retention times for labetalol and propranolol were 2.7 and 3.3 min, respectively. The detection limit by this method was 10 ng of labetalol per mL plasma. The CV was 4-6% for 10-100 μg/mL labetalol

Using the above procedure Wood et al. [106] found that the distribution and elimination kinetic parameters of labetalol in patients with severe renal failure were similar to those in normal controls ($t_{\frac{1}{2}}$ = 3.3-7.4 hr; clearance = 0.5-1.6 L/hr/kg).

Another procedure for measurement of labetalol involves ion-pair reversed-phase chromatography (10 μm LiChrosorb 10 RP-18, elution with 32:68 (v/v) CH_3CN:aqueous solution of 40 g trimethylammonium chloride, 4 g NaOAc, and 40 g $NaClO_4$ per liter; adjusted to pH 9.3 with borate buffer). Detection was accomplished by using a fluorescence detector (excitation at 335 nm, emission at 370 nm). This method had a detection limit of 1 ng/mL [107]. Other HPLC methods [108-110] have also been described for the measurement of labetalol.

E. Acebutolol

After oral administration of acebutolol about one-half of the dose is absorbed. Its plasma $t_{\frac{1}{2}}$ is in the range of 3-8 hr. The major metabolite of acebutolol is diacetolol, which has activity similar to that of the parent drug. During chronic oral acebutolol therapy, the circulating levels of the metabolite exceed that of the unchanged drug.

Acebutolol in plasma has been measured by an HPLC method using the extraction procedure of Holt et al. [74] similar to that described for propranolol (see earlier). The propionamide analogue of acebutolol (MB 17764) was used as the internal standard. Samples were injected on a 5-μm Spherisorb S5 ODS column (10 cm × 0.46 cm). Elution was carried out with a mobile phase consisting of a 7:3:1 v/v mixture of 0.01 phosphate buffer, pH 4:MeOH:CH_3CN. A 5-cm guard column (Whatman CO-Pell ODS) protected the main column. Detection was achieved by the measurement of the native fluorescence (excitation at 254 nm and emission at 460 nm). The coefficient of variation for acebutolol added to plasma was 5.3% at 0.2 μg/mL and 2.0% at 1.0 μg/mL. The detection limit of the method is 10 ng/mL acebutolol in plasma.

Lefebvre et al. [55] used a μBondapak C_{18} column and a mobile phase consisting of MeOH:AcOH:H_2O (50:1:49 v/v/v) pumped at a flow-rate of 1.3 mL/min to quantitate acebutolol and diacetolol. A fluorescence detector (excitation at 235 nm and emission at 389 nm) was used to monitor the effluent. These authors have described optimal conditions for extracting the drug and metabolite from plasma.

Zaman et al. [111] measured the concentration of acebutolol and diacetolol in plasma, saliva, and human cerebrospinal fluid by HPLC with

UV detection at 254 nm. This method was an adaptation of that reported by Meffin et al. [112], and utilized extraction conditions reported by Emerson et al. [113]. In this study, the CSF/plasma and saliva/plasma ratios for the metabolite were one-third those of the drug, and this may reflect the greater polarity of diacetolol, restricting its passages across membranes. The studies suggested that the metabolic conversion of acebutolol to diacetolol is not responsible for any interpatient variations seen in the clinical response to oral acebutolol. Other HPLC methods [56,114-117] have been described for the measurement of acebutolol.

F. Pindolol

Pindolol, a nonselective beta blocker, is almost completely (>95%) and rapidly absorbed. Its bioavailability is about 90% of the dose. Plasma $t_{\frac{1}{2}}$ has been reported to be in the range of 2-5 hr. About 60% of the dose is metabolized, and the remainder is excreted unchanged in the urine. The metabolites are inactive. Aging does not modify the disposition of pindolol [85].

Measurement of plasma pindolol levels involves extraction of alkalinized plasma (1 mL plus 0.1 mL of 0.1 N NaOH) with 7 mL of $CHCl_3$:*n*-pentanol (60:20, v/v). The organic phase is extracted with 0.3 mL 0.1 N H_2SO_4, and aliquots of the aqueous phase are separated on μBondapak C_{18} column (30 cm × 0.4 cm) by a mobile phase (MeOH:AcOH:H_2O, 40:1:49, v/v/v) pumped at a flow-rate of 1.3 mL/min. Detection is achieved at 280 nm. Pindolol and oxprenolol (used as internal standard) elute at 4.2 and 6.6 min, respectively. The detection limit is 20 ng/mL of pindolol in plasma [55]. Other HPLC methods have been described [56,118] for the quantitation of pindolol.

G. Timolol

Timolol is a nonselective beta blocker. When given orally, it is fairly completely (>90%) absorbed; its systematic bioavailability is 60-75% of the dose. Peak plasma levels are obtained in about 1 hr, and levels decline with a $t_{\frac{1}{2}}$ of 2-4 hr. About 80% of the dose is metabolized; the metabolites are inactive.

Timolol in plasma (1 mL, alkalinized with 0.1 mL of 0.1 N NaOH) was extracted with 7 mL of $CHCl_3$:*n*-pentanol (60:40, v/v), and the organic phase was reextracted into 0.3 mL of 0.1 N H_2SO_4. The aqueous phase was injected on a 30 cm × 0.3 cm μBondapak C_{18} column. The mobile phase was MeOH:AcOH:H_2O (50:1:49, v/v) pumped at a flow-rate of 1.3 mL/min. The UV detector was set at 295 nm. Timolol and the internal standard eluted at 4.0 and 6.4 min, respectively. The detection limit was 40 ng/mL timolol in plasma [55].

H. Sotalol

Sotalol is a nonselective beta blocker. The bioavailability of an oral dose of sotalol is about 90%; peak plasma levels are achieved in 2-4 hr. Plasma $t_{\frac{1}{2}}$ is in the range of 5-18 hr. Patients with renal impairment have decreased clearance, and increased plasma $t_{\frac{1}{2}}$ [119].

For the analysis of sotalol, plasma or urine (1 mL) was mixed with 1 mL of CH_3CN and solid K_2CO_3 (0.5 g) was added. After shaking and centrifugation, the organic layer was separated and dried (N_2). The residue was dissolved in MeOH (0.1 mL) and aliquots of this solution were injected on a μBondapak C_{18} reversed-phase column. Elution was carried out with a solvent consisting of 160 mL CH_3CN, 5 mL AcOH, and 840 mL of 0.005 M sodium 1-octanesulfonic acid, pumped at 2 mL/min. The detector was set at 232 nm. The retention time for sotalol was 7.3 min [119].

I. Nadolol

Nadolol is a nonselective beta blocker. After oral administration, the drug is poorly absorbed (<35%); maximum plasma levels are achieved in 2-3 hr. Plasma $t_{\frac{1}{2}}$ of nadolol is in the range of 14-24 hr. The drug is not significantly metabolized.

The diasteroisomers of nadolol were quantitated by HPLC [120]. An HPLC method has been described for the quantitation of nadolol in drug formulations and bulk material [56]. The drug was extracted with the mobile phase and injected on a 10-μm reversed-phase ethylsilane (RP-2) column (25 cm × 0.46 cm) and eluted with 35:65 v/v MeOH:aq. 0.0005 M HCl – 0.05 M NaCl pumped at 1-2 mL/min. The detector was set at 220 nm. Atenolol was used as the internal standard.

J. Oxprenolol

About 75-90% of an oral dose of oxprenolol is absorbed, but bioavailability is low due to high first pass effect (plasma $t_{\frac{1}{2}}$ = 1-4 hr). The HPLC procedure for nadolol [56] can be applied for the measurement of oxprenolol in drug formulations. The enantiomers of oxprenolol have been separated by HPLC [90,121].

K. Alprenolol

Alprenolol is a nonselective beta blocker. Orally administered alprenolol has a bioavailability of 20-50% and plasma $t_{\frac{1}{2}}$ of 2-3 hr. The *R*- and *S*-isomers of alprenolol have been separated by HPLC [90,97]. Alprenolol levels in dosage formulation can be determined by the procedure used for nadolol [56].

L. Practolol

Practolol is no longer used. HPLC methods for the measurement of this cardioselective beta blocker have been described [56,74,122,123].

V. DIURETICS

Several types of diuretics are commonly used today. They include thiazides, thiazide-like derivatives, loop diuretics, and potassium-sparing diuretics. Thiazides are the most commonly used diuretics. The mechanism by which they produce an antihypertensive effect is not entirely clear. Their diuretic effect is produced by inhibition of sodium reabsorption in the cortical ascending limb of the loop of Henle and the distal convoluted tubule. This action results in an acute fall in cardiac output and decrease in extracellular fluid volume, plasma volume, and blood pressure. After weeks of treatment, the cardiac output returns to baseline levels despite persistent lowering of blood pressure and reduced peripheral resistance. A chronically decreased plasma volume and/or extracellular fluid volume appears to be, at least in part, responsible for the persistent antihypertensive effect.

The loop diuretics inhibit the reabsorption of chloride and sodium in the medullary portion and the ascending limb of the loop of Henle. The mechanism of antihypertensive action of loop diuretics is probably similar to that of thiazides.

The potassium-sparing agents act solely on the distal convoluted tubule, where they affect only a small percentage of filtered sodium and water, and consequently are weak diuretics. At this site, however, potassium secretion into the renal tubule is also blocked by these compounds. The potassium-sparing diuretics are generally used in combination with thiazide or loop diuretics to help prevent hypokalemia, caused by the latter agents.

A. Thiazides

The benzothiazide ("thiazide") diuretics are widely used for the treatment of hypertension, congestive heart failure, and other edematous conditions. All thiazides augment excretion of potassium (in the distal tubule) and can cause hypokalemia in patients taking them without potassium supplements for a prolonged period. The duration of action of thiazides ranges between 6 and 18 hr, and longer for polythiazide and chlorthalidone.

The HPLC methods for measurement of these diuretics are more rapid, simple, and sensitive than other procedures, such as spectrophotometric, TLC, or GC methods.

Barbhaiya et al. [124,125] measured the levels of chlorothiazide and hydrochlorothiazide in plasma and urine of healthy subjects after

oral administration of 500 mg of chlorothiazide or 50 mg of hydrochlorothiazide. Plasma (2 mL) was mixed with 0.1 mL of internal standard (40 μg hydroflumethiazide) and extracted with 4 mL of toluene. The aqueous layer was mixed with 0.5 mL of 0.01 M acetate buffer (pH 3.8) and extracted with 5 mL of EtOAc. The organic layer was evaporated to dryness and the residue was reconstituted in 75 μL of MeOH. Then 20 μL was injected into the chromatograph (30 cm × 0.4 cm reversed-phase octadecyl column). The mobile phase consisted of 15% CH_3OH in 0.01 M AcOH pumped at a flow-rate of 2.5 mL/min; detection was at 228 nm. Chlorothiazide and hydrochlorothiazide eluted at 3.5 and 4.6 min, respectively.

Urine was extracted and assayed for the two drugs by a slightly modified procedure. The sensitivity of the method was 1 ng/mL. Peak plasma levels of 420-850 ng/mL were achieved 1-2 hr following the dose of chlorothiazide, and 450-550 ng/mL at 2-3 hr after the dose of hydrochlorothiazide. Plasma $t_{\frac{1}{2}}$ was in the range of 8-12 hr for hydrochlorothiazide. The decline of plasma levels of chlorothiazide followed a triphasic pattern, $t_{\frac{1}{2}}\gamma$ = 15-27 hr [126].

Hydrochlorothiazide was assayed in tablet formulations and in the bulk form by HPLC after extraction with MeOH [127]. The extract was injected on a 10-μm μBondapak C_{18} column (30 cm × 0.4 cm); elution was carried out with 5% (v/v) MeOH in H_2O at pH 4.5-5.0 (adjusted with 0.1 M AcOH) at a flow-rate of 2 mL/min. The detector was set at 254 nm. The retention times for hydrochlorothiazide and sulfadiazine (internal standard) were 13.5 and 16.5 min, respectively. Chlorothiazide was also separated by this method (retention time = 12 min).

Tisdall et al. [128] developed an HPLC procedure to detect the presence of a number of thiazides in urine: chlorothiazide, hydrochlorothiazide, bendroflumethiazide, methylclothiazide, cyclothiazide, polythiazide, trichlormethiazide, hydrofluthiazide, and benzthiazide.

Urine was treated with sodium borohydride to reduce chlorothiazide to hydrochlorothiazide. After acidification with 1 M NaH_2PO_4 (pH 5), the thiazides were extracted with EtOAc. Interfering substances were removed by washing the organic layer twice with 0.1 M NaH_2PO_4 (pH 8). The solvent was evaporated and the residue was dissolved in the mobile phase, and aliquots were injected on a μBondapak C_{18} (30 cm × 0.4 cm) reversed-phase column. Two mobile phases (2 mL/min) were used to separate all of the thiazides: (a) 8:92 v/v CH_3CN:AcOH (0.1% by volume in water) and (b) 35:65 v/v CH_3CN:AcOH (0.1%). A UV detector set at 271 nm was used to monitor the effluent. The recovery of thiazides from urine ranged from 53 to 93%. All of the thiazides were detected in the urine of patients and volunteers given the diuretics [128]. Several other HPLC methods have been described for the measurement of chlorothiazide [129-134] and hydrochlorothiazide [135-141].

B. Chlorthalidone

After oral administration, systematic bioavailability of chlorthalidone, a thiazide-like diuretic, is about 60%. The diuretic has a relatively long plasma $t_{\frac{1}{2}}$ (approximately 45 hr) and correspondingly long duration of effect.

Chlorthalidone, in whole blood, was measured by an HPLC method [142]. Blood (0.2 mL) was diluted with distilled H_2O (0.2 mL), sonicated for 5 min, and then mixed with CH_3CN (0.5 mL) containing phentolamine hydrochloride as internal standard. After mixing and centrifugation, the supernate was transferred to a clean tube and evaporated (N_2) until about 0.4 mL of the solution remained. A portion of this sample was injected directly on the column. Urine was handled as blood, with the exception that the sonication and centrifugation steps were not included and the internal standard was sodium pentobarbital. Chromatography was performed on a Perkin-Elmer Series 3 chromatograph equipped with a variable-wavelength spectrophotometric detector. The UV detector was set at 210 nm for blood and 250 nm for urine samples. The lower limit of sensitivity of the assay in blood was 200 ng/mL with a CV of 5%, in urine, 750 ng/mL with a CV of 6.6%. The drug was given as two 25-mg tables to 22 healthy male volunteers. The $t_{\frac{1}{2}}$ of absorption was 7.73 hr, and the $t_{\frac{1}{2}}$ of elimination was 46.7 ± 22.2 hr. The absorption was slow; maximum blood levels of 4.62 ± 0.8 μg/mL were obtained at 10.8 ± 5 hr. Chlorthalidone concentrates in red cells and plasma levels are low.

Recently, a method has been described for determination of the stability of chlorthalidone in tablets [143]. The drug was extracted with acetone. The solution was filtered and then evaporated to dryness. The residue was dissolved in acidic MeOH, and aliquots of the solution were applied to μBondapak C_{18} column. The column was eluted with a 30:70 v/v mixture of CH_3CN:2% AcOH, pumped at a flow-rate of 1.5 mL/min. The detector was set at 280 nm. Chlorthalidone and the internal standard (*p*-nitroaniline) eluted at 3.9 and 5.9 min, respectively.

Several other HPLC methods have been reported for the measurement of chlorthalidone [21,144].

C. Furosemide

Furosemide, a potent loop diuretic, is fairly rapidly absorbed after oral administration; bioavailability ranges from 60 to 70%. Its plasma $t_{\frac{1}{2}}$ is about 1-2 hr. and the duration of action is 6-8 hr. Furosemide is eliminated by the kidney almost entirely via proximal tubular secretion. In normal subjects, about one-half of a dose of furosemide is eliminated by nonrenal processes such as biotransformation and biliary excretion [145,146].

Furosemide concentration in plasma and urine was determine fluorometrically after HPLC separation [146]. Plasma (0.25 mL) was diluted

with 0.25 mL of phosphate buffer, pH 7.0, and the mixture was extracted with 5 mL of Et_2O; the organic layer was discarded. The aqueous layer was acidified with 50 μL of 5 N HCl, and furosemide was extracted with 5 mL Et_2O. The Et_2O layer was evaporated (N_2) and the residue was dissolved in 0.1 mL of phosphate buffer and assayed. The phenyl analogue of furosemide was used as the internal standard (100 ng/mL plasma). Urine was assayed directly after centrifugation.

The extracts of plasma or urine were separated on a 12 cm × 0.43 cm column packed with 5 μm reversed-phase irregular, organically modified silica. The mobile phase consisted of MeOH:0.1 M H_3PO_4 (53:47, v/v). A fluorescence detector (excitation at 233 nm and emission at 370 nm) monitored the eluent. The standard curve was found to be linear over the concentration range 10-1000 ng/mL for plasma and 0.5-10 μg/mL for urine.

Using the above HPLC method, it was determined that the absorption of furosemide from sustained-release tablets (60 mg) was slower than from conventional tables (40 mg). The 24-hr water and electrolyte excretion for slow-release preparation was less than that obtained with the conventional tablets. However, the 48-hr values were not different for the two dosage forms [146].

In another procedure [147,148] sodium phenobarbital (internal standard, 50 μL of 0.05%) was added to 200 μL of plasma prior to precipitation of proteins with CH_3CN (400 μL). After mixing and centrifugation, the supernatant fraction was concentrated (N_2) and injected on a μBondapak C_{18} reversed-phase column (30 cm × 0.4 cm). Urine samples were mixed with the internal standard and injected directly after centrifugation. The mobile phase consisted of 38% CH_3CN-0.015 M H_3PO_4 and was pumped at a flow-rate of 2 mL/min. Detection was achieved with a fluorescence detector (excitation at 345 nm, emission at 405 nm). The retention times for furosemide and phenobarbital were 6 and 4 min, respectively. The detection limit was 8 ng/mL for furosemide in plasma.

Using the HPLC procedure of Carr et al. [149], it was determined that the elimination of furosemide from plasma was somewhat prolonged in cirrhotic patients ($t_{\frac{1}{2}}$ = 60.2 ± 5.8 min in controls vs. 81.0 ± 8.0 min in patients), but the systemic clearance (156 ± 7 mL/min) was not affected [150]. The difference in $t_{\frac{1}{2}}$ was due to the increased percentage of free furosemide in plasma of cirrhotics (10.2 ± 1.0%) as compared to normals (4.0 ± 1.0%). There was no difference in urinary excretion of unchanged drug (58.8 ± 2.8%) or glucuronide metabolite (17.8 ± 1.5%).

Fuller et al. [151] reported doubling of plasma $t_{\frac{1}{2}}$ of furosemide in patients with hepatic cirrhosis with ascites. The $t_{\frac{1}{2}}$ of the diuretic was also prolonged in congestive heart failure, uremia, and other edematous states [152-157]. Furosemide disposition is altered (decreased clearance, increased $t_{\frac{1}{2}}$) in the elderly [158].

Many other HPLC procedures have been described in the literature for the measurement of furosemide and its metabolites [159-163].

D. Bumetanide

Bumetanide is a loop diuretic with a pharmacological action similar to that of furosemide, but it is 40-60 times more potent than furosemide. The bioavailability of an oral dose of bumetanide is about 90%. After i.v. administration, bumetanide is eliminated from plasma in a triphasic pattern ($t_{\frac{1}{2}}\alpha$ = 6 min, $t_{\frac{1}{2}}\beta$ = 46 min, $t_{\frac{1}{2}}\gamma$ = 3 hr). Plasma $t_{\frac{1}{2}}$ of an oral dose ranges between 1 and 2 hr. About 40-50% of the dose is metabolized.

The drug has been measured by fluorometric, GC, radioisotopic, radioimmunassay, and HPLC methods (see Ref. 164). Recently, a rapid, sensitive, and specific HPLC method was developed by Smith [164] for the measurement of the diuretic in plasma and urine. Plasma (0.2 mL) containing the internal standard (acetophenone, 12.5 μg) was treated with CH_3CN (0.4 mL) to precipitate the proteins. After mixing and centrifugation, the supernatant fraction was injected directly on a Partisil-10 ODS-3 reversed-phase column (25 cm × 0.46 cm). The mobile phase consisted of MeOH:H_2O:AcOH (70:30:1, v/v) pumped isocratically at a flow-rate of 1.5 mL/min at ambient temperature. A fluorescence detector (excitation 338 nm, emission 433 nm) was used for bumetanide, and a UV detector (254 nm) for simultaneous detection of the internal standard. Other HPLC methods for the analysis of bumetanide have been reported [165,166].

E. Indapamide

The antihypertensive effect of indapamide, a nonthiazide sulfonamide, is produced by lowering of peripheral vascular resistance as a result of a decrease in arteriolar hyperreactivity to vasopressor amines. Indapamide has a prolonged blood pressure-lowering effect.

After oral administration, the drug is rapidly and completely absorbed. Elimination from plasma follows a biphasic pattern ($t_{\frac{1}{2}}\beta$ = 14 hr, $t_{\frac{1}{2}}\gamma$ = 25 hr). Indapamide is extensively metabolized, giving rise to a large number of metabolites (see Ref. 167,168). The drug accumulates in the vascular wall of blood vessels, and its plasma levels are quite low.

Indapamide in bulk material was measured by HPLC [169] using a 10-μm μBondapak C_{18} (30 cm × 0.4 cm) column. Sulfizoxazole was used as the internal standard. The mobile phase, consisting of CH_3CN:H_2O, 35:65 at pH 2.8, was pumped at 2 mL/min. The detector was set at 254 nm. Indapamide, the internal standard, and one of the metabolites eluted at 6.8, 3.5, and 2.5 min, respectively.

Choi et al. [170] have described procedures for quantitating indapamide in blood, plasma, and urine. Blood (1 mL) was mixed with the internal standard, sulfabenz (sulfanilanilide), and extracted twice with Et_2O (4 mL × 2). The organic phase was reextracted with 0.5 mL of 0.01 N NaOH. The alkaline solution was neutralized (0.5 mL 0.01 N HCl plus 0.25 mL 0.05 M phosphate buffer, (pH 7.4) and then extracted with Et_2O (4 mL). The Et_2O phase was evaporated to dryness. The residue was taken in MeOH and analyzed on a LiChrosorb C_{18} (25 cm × 0.32 cm) column maintained at 54°C. The mobile phase, CH_3CN:0.1 M NaOAc buffer (35:65, pH 3.6) was pumped at 1.5 mL/min. The detector was set at 241 nm. The sensitivity of the method was 50 ng/mL in blood and urine and 25 ng/mL in plasma.

F. Spironolactone

Spironolactone acts primarily through competitive binding of receptors at the aldosterone-dependent sodium-potassium exchange site in the distal convoluted tubule. The drug causes increased amounts of sodium and water to be excreted, while potassium is retained.

Spironolactone, given orally, is well absorbed (>90%). It is rapidly and extensively metabolized. The primary metabolite, canrenone, is active and attains peak plasma levels at 2-4 hr following a single oral dose. The decline of plasma canrenone levels is biphasic ($t_{\frac{1}{2}}\beta$ = 12.5 ± 3.4 hr; $t_{\frac{1}{2}}\gamma$ = 54 ± 6 hr). The $t_{\frac{1}{2}}\beta$ of canrenone is prolonged (19.2 ± 6.6 hr) after chronic oral dosing.

Canrenone has been measured in plasma and urine by HPLC [171] on a 10-μm LiChrosorb SI-100 (25 cm × 0.25 cm) column. Plasma or urine (1 mL) was extracted with 0.2 mL of $CHCl_3$, and the organic phase was injected on the column. The mobile phase, consisting of a 50:50 mixture of $CHCl_3$:*n*-hexane was pumped at 2 mL/min. The detector was set at 283 nm. Canrenone eluted at 4.5 min. Other HPLC methods have been described for the measurement of canrenone [172,173].

G. Triamterene

Triamterene is a widely used mild diuretic. The drug inhibits the reabsorption of sodium in exchange for potassium and hydrogen in the distal tubule. Generally, it is combined with other diuretics to minimize their kaliuretic side effects.

After oral administration, peak plasma levels of triamterene are achieved in 1-3 hr. Plasma levels decline in a biphasic pattern; $t_{\frac{1}{2}}\beta$ = 5 hr. Triamterene is extensively metabolized. The major metabolite (about 80% of the dose), the sulfate conjugate of *p*-hydroxytriamterene, is active [174].

Several HPLC methods have been described for the measurement of triamterene and *p*-hydroxytriamterene [141,175-178].

Sved and co-workers [175,176] extracted triamterene as its perchlorate ion pair (0.25 mL 3 M $HClO_4$ added to 0.5 mL plasma or urine) with methyl isobutylketone (1 mL). Aliquots (50 μL) of the supernatant fraction were injected on a 25 cm × 0.32 cm LiChrosorb Si 60 5-μm column. The mobile phase, consisting of CH_2Cl_2:hexane: MeOH:70% $HClO_4$ (57:35:8:0.1) was pumped at 2 mL/min. A fluorescence detector (excitation at 335 nm, emission at 470 nm) monitored the effluent. Triamterene and metabolite eluted at 3.5 and 5.0 min, respectively. Detection limit was 0.25 ng/mL.

Brodie et al. [177] used methoxytriamterene as the internal standard and extracted the diuretic from plasma (1 mL, with 200 mg $NaHCO_3$) with EtOAc (5 mL). The organic phase was evaporated to dryness (N_2), and the residue was reconstituted with MeOH. Urine (0.1 mL) diluted with 1 mL of H_2O was extracted in a similar way. The MeOH solution was injected on a μBondapak C_{18} 10-μm column (30 cm × 0.4 cm). The mobile phase consisting of 45% v/v MeOH in aqueous 0.1% w/v KH_2PO_4, pH 3.8 (adjusted with H_3PO_4), was pumped at 2 mL/min. Detection was by measurement of fluorescence (excitation 365 nm, emission 440 nm) or UV absorption (254 nm). Triamterene and the internal standard eluted at 2.6 and 3.1 min, respectively. The sensitivity, 10 ng/mL with UV and 1 ng/mL with fluorescence detection, was less than that in the above described procedure.

A rapid and selective method to measure triamterene and *p*-hydroxytriamterene was developed by Yakatan and Cruz [178]. Plasma (0.5 mL) to which the internal standard (4,7-diamino-2-dimethylamino-6-phenylpteridine) had been added was extracted with 10 mL of Et_2O-isopropyl alcohol (95:5). The organic phase was evaporated (N_2), and the residue was dissolved in the mobile phase (CH_3CN:H_2O:AcOH, 60:39.5:0.5). The solution was analyzed on a reversed-phase column with UV detection (365 nm). The sensitivity of the method (20 ng/mL) was lower than in the procedures described earlier.

HPLC methods have been described for the measurement of many other diuretics: bemetizide [179], bendrofluazide [180], bendroflumethiazid [181,182], benzthiazide [183], cyclothiazide [181], hydroflumethiazide [184,185], methylclothiazide [186], polythiazide [35,187-189], trichlormethiazide [190,191], tricrynafen [192], and tripamide [193].

VI. INHIBITORS OF THE RENIN-ANGIOTENSIN SYSTEM

Several compounds have been introduced as antihypertensive agents which competetively inhibit the angiotensin-converting enzyme (ACE) that converts angiotensin I, an inactive substance, to angiotensin II, a powerful vasoconstrictor also associated with aldosterone stimulation. Animal and human studies confirm that ACE inhibitors block the generation of angiotensin II and raise renin levels. They also decrease

aldosterone production and induce a negative sodium as well as a positive potassium balance which may be significant in the management of hypertension.

Captopril was the first orally active ACE inhibitor to become available for clinical use. It is a potent antihypertensive agent, being of special value in many types of high renin hypertension.

After oral administration, captopril is rapidly and fairly well absorbed (75%); 50 mg of the drug given to four patients produced peak plasma levels of 297 ± 133 ng/mL in about 75 min [195]. Plasma $t_{\frac{1}{2}}$ of captopril is in the range of 1.6-2.7 hr. About 50% of the absorbed drug is metabolized to the disulfide dimer of captopril and captopril cysteine disulfide.

An assay for the drug in plasma may be of value in optimizing the dosages, especially as there is evidence that side effects of the drug may be dose related. Methods other than the HPLC procedure are cumbersome since they involve extraction and derivatization prior to assay (see Ref. 194).

Captopril in plasma and urine has been measured as follows [194]: freshly drawn heparinized blood was centrifuged at 2000 × *g* for 5 min at 4°C to obtain plasma. Protein was precipitated by mixing 100 μL (25 mg) of sulfosalicylic acid with 1 mL of plasma followed by centrifugation (5 min). The protein-free plasma (which can be stored at −20°C until analyzed) was again spun at 2000 × *g* for 5 min. Urine, collected in 6 M HCl (1 mL/100 mL urine), was centrifuged for 5 min at 200 × *g*. Then 10 to 25-μL aliquots of the supernatant fraction of plasma or urine were injected on a 10 cm × 0.5 cm 5-μm ODS-Hypersil column. Elution was carried out with a mobile phase consisting of a 35:65 v/v mixture of MeOH and 100 mM KH_2PO_4 adjusted to pH 2 (with H_3PO_4) and pumped at a flow-rate of 1 mL/min. An EC detector was employed with Au/Hg cell (+ 0.07 V) and Ag/AgCl as reference electrode. This procedure can detect as little as 0.5 pmol of the drug in plasma or urine.

Another method is described by Shimada et al. [195] who used a 30 cm × 0.4 cm μBondapak C_{18} column and a mobile phase consisting of 1:2 v/v CH_3CN:0.8% $NH_4H_2PO_4$ (pH 3.0). These investigators also used EC detection; however, captopril was derivatized with *N*-(4-dimethylaminophenyl)maleimide prior to injection of the samples on the column. The detection limit was 10 ng/mL in blood.

Perlman and Kirschbaum [196] tested several columns (anion exchange, amine, and reversed-phase octadecylsilane) to find optimum conditions for quantitating captopril in the presence of precursors and intermediates in the synthesis of the drug and the disulfide metabolite. The reversed-phase column was found to be the best in this series. Other HPLC methods have been described for quantitation of captopril [197,198].

VII. VASODILATORS

Vasodilators cause relaxation of smooth muscle in the vascular bed. Some of these agents act predominantly on the arterioles and venules. Vasodilation of the arterial bed decreases total peripheral resistance and arterial blood pressure.

A. Hydralazine

Hydralazine (HP), a potent, peripherally acting vasodilator, is widely used in the treatment of essential hypertension. Because of its side effects (sympathetically mediated reflex tachycardia and increase in cardiac output), HP is often given with a beta blocker. HP is also combined with reserpine and diuretics since the latter agents potentiate the hypotensive effect of HP. The mode of action of HP involves reduction of vascular resistance through direct relaxation of arteriolar smooth muscle.

The bioavailability of orally administered HP depends on acetylator phenotype and is estimated to be 7-10% in rapid and 31-39% in slow acetylators [199,200]. Using specific HPLC assays, it was determined [200,201] that the $t_{\frac{1}{2}}\beta$ of HP is independent of acetylator phenotype (<0.7 hr after i.v. dose and <0.5 hr after an oral dose). However, the pattern of metabolism is different in slow and fast acetylators [201-203].

In man, HP is metabolized extensively, giving rise to a large number of metabolites [203-206]. Some of the metabolites possess antihypertensive properties. A major pathway of metabolism is via acetylation, which follows a polymorphic pattern.

The duration of hypotensive effect of HP (during chronic administration) far exceeds that predicted from the plasma $t_{\frac{1}{2}}$ of the drug [207]. This may be due to accumulation of HP (or active metabolites) in a deep compartment, such as the walls of the blood vessels and slow release therein [46,208].

A number of methods have been developed to measure HP and its metabolites. These include colorimetric, spectrophotometric, spectrophotofluorometric, polarographic, inverse isotope dilution, gas chromatographic, and HPLC procedures (see Refs. 204-206).

Many of the early assay procedures and even some of the recently described techniques for the measurement of HP suffer from interference by acid-labile metabolites and measure only apparent HP levels. Furthermore, reaction of HP with α-ketoacids in plasma, particularly with pyruvic acid, continues in the blood after it is drawn. Thus, many of the pharmacokinetic parameters determined using nonspecific HP assays may be misleading [200,205,209,210].

Reece et al. [200] and Ludden et al. [211] employed conditions that avoid hydrolysis of acid-labile metabolites and conversion of HP to hydrazones. For the measurement of HP in plasma, Reece et al. [200] transferred 8-12 mL of venous blood into ice-cold polypropyl-

ene tubes containing 125 IU of lithium heparin. After mixing, the samples were rapidly centrifuged (30 sec), and the supernate was immediately transferred into cold tubes. Aliquots (1 mL) were mixed with 0.075 mL of aq. $NaNO_2$ (50%), the internal standard (0.15 mL of 16.7 μM 4-methyl-HP), and 2 mL of 0.02 N HCl (final pH = 5.5). After vortexing, the mixture was allowed to stand for 10 min at 20 ± 1°C. (The entire procedure from the time of drawing blood to the time of adding the acid was performed in less than 5 min.) Then 1 mL of 1 N NaOH - 0.6 M sodium tetraborate (pH 10) was added, and the alkalinized solution was extracted with $CHCl_3$. The organic layer was evaporated (N_2, 45°C), and the residue was reconstituted in 0.5 mL of the mobile phase. Aliquots of the final solution were injected on a 10-μm alkyl phenyl reversed-phase column (μBondapak/phenyl). The column, maintained at 50°C, was eluted with a 85:15 v/v mixture of 1.5 mM H_3PO_4:CH_3CN, pumped at 2 mL/min. The column effluent was monitored using a fluorescence detector (excitation at 250 nm, cutoff filter for emission at 360 nm). The retention times for the derivatives of HP and the internal standard were 2 and 11.5 min, respectively. The detection limit was 1 ng/mL. These authors also quantitated a number of metabolites of HP by modifying the above method.

Recently, Ludden et al. [211] described a procedure which determines HP in human whole blood, circumventing the problem of drug degradation in sample handling. HP in whole blood (3 mL) containing EDTA and 4-methylhydralazine (as internal standard) was derivatized with *p*-anisaldehyde (40 ng) at room temperature for 10 min. The hydrazones of HP and the internal standard were extracted with hexane (10 mL). The organic phase was evaporated to dryness (N_2), and the residue was taken up in 0.1 mL of MeOH and injected on μBondapak CN column. Elution was carried out with 70% (v/v) CH_3CN in 0.15 M NaOAc buffer, pH 3.0, pumped at a flow-rate of 2 mL/min. Detection was at 365 nm. The retention times for the hydrazones of HP and internal standards were 3.5 and 6 min, respectively. Maximum sensitivity = 3 ng/3 mL blood.

Facchini and co-workers [212,213] measured several metabolites of HP in urine of subjects who received the drug. Urine (10 mL) was spiked with the internal standard (methylphthalazinone), adjusted to pH 9.5, and extracted with CH_2Cl_2 (30 mL). The organic layer was filtered and then evaporated to dryness. The residue was dissolved in MeOH (1 mL) and applied to a 25 cm × 0.5 cm reversed-phase ODS-Hypersil column. The mobile phase, MeOH:H_2O (15:85, v/v), was pumped at a flow-rate of 1.9 mL/min. The metabolites and internal standard (detected by UV absorption at 254 nm) eluted at 15, 26, and 37 min, respectively. The sensitivity of the method was 0.5 μg/mL. Other HPLC methods have been described for the measurement of HP [214-218] and similar drugs, dihydralazine [219], and endralazine [220,221].

B. Minoxidil

Minoxidil is a potent, long-acting peripheral vasodilator. Because of its side effects, the drug is used in controlling severe hypertension in patients refractory to maximum tolerated doses of conventional antihypertensive agents, including hydralazine. The drug is well absorbed (>90%) when given orally, and peak plasma levels are achieved rapidly (0.5-1 hr). It was a $t_{\frac{1}{2}}$ of about 4 hr. Minoxidil is localized in tissues (e.g., arterial walls) and thus has a V_d of four times the total body water. It is extensively metabolized, mainly by glucuronidation; some of the metabolites are active. Like hydralazine, the extent and time course of blood pressure reduction by minoxidil does not correlate with its plasma levels. The hypotensive effect of minoxidil often persists for 24 hr or more. No HPLC methods have been described for the measurement of minoxidil.

C. Diazoxide

Diazoxide, a potent antihypertensive agent, decreases blood pressure by lowering peripheral resistance (presumably by direct effect on arteriolar smooth muscle). The drug is eliminated from plasma with a $t_{\frac{1}{2}}$ of 20-36 hr, and is extensively metabolized. The metabolites are inactive. The duration of action of the drug is shorter than its plasma $t_{\frac{1}{2}}$.

Several spectroscopic, GC, and GC-MS methods have been described for the analysis of diazoxide in plasma and urine (see Ref. 222). Vree et al. [222] developed a simple and rapid HPLC procedure for measuring diazoxide in biological fluids. Plasma (0.1-1 mL) or urine (0.01 mL) was mixed with 0.4-0.5 mL of $HClO_4$ (0.33N) and centrifuged. The supernatant liquid (0.1 mL) was injected directly on a 5-μm Lichosorb RP-8 (10 cm × 0.46 cm) column. The column was eluted with MeOH: H_2O (2:3 v/v) pumped at a flow-rate of 1 mL/min; detection was at 270 nm. The retention time of diazoxide was 3.5 min, and the limit of detection was 0.1 μg/mL.

D. Sodium Nitroprusside

Sodium nitroprusside is a potent smooth muscle relaxant which causes vasodilation of both venous and arterial vascular beds. It is administered intravenously, mostly in hypertensive crises since it lowers blood pressure in seconds. Because of the instability of the solution of the drug, many methods do not measure it reliably and reproducibly.

Baaske et al. [223] measured sodium nitroprusside in commercial lyophilized preparation or in i.v. solutions by HPLC. The solution of the drug in 5% aqueous dextrose was inejcted on a 30 cm × 0.4 cm column packed with phenyl bonded to 10-μm silica gel. Elution was carried out with a mixture consisting of 300 mL of CH_3CN and 700 mL

of 0.01 M K_2HPO_4 and 0.005 M tetrabutylammonium hydroxide adjusted to pH 7.1 (H_3PO_4). The solvent was pumped at a flow-rate of 2 mL/min. The detector wavelength was set at 210 nm. The drug eluted at 6 min, while most of the photodegradation products eluted within 3 min of injection. These authors showed that there was a small loss of nitroprusside due to photodegradation during I.V. infusion, even when the admixture container is wrapped in foil as recommended, and used expeditiously.

VIII. DRUGS WITH MISCELLANEOUS MODES AND SITES OF ACTION

A. Calcium Antagonists

The calcium entry blockers (calcium antagonists) influence the cardiovascular system through inhibition of calcium influx into cell plasma. These agents produce coronary, venous, and arterial dilation and a negative ionotropic effect. The primary hemodynamic effect of calcium blockers is a reduction of total peripheral resistance with a subsequent decrease of blood pressure. The role of calcium blockers in the chronic management of essential hypertension has not been established. Some of the newer calcium blockers which are modifiers of calcium metabolism have been called curative in hypertension [224].

1. Nifedipine

Nifedipine, a potent and long-lasting vasodilator, is well absorbed (>90%) after oral or buccal administration. Its systemic bioavailability varies widely (56 ± 25%) in different individuals. Its plasma $t_{\frac{1}{2}}$ is in the range of 1.5-5.0 hr. The drug undergoes extensive presystemic metabolism, and decomposes rapidly upon exposure to visible or UV light.

In biological fluids, nifedipine and its metabolites were measured by fluorescence, TLC (of labeled drugs), GC-MS, GC-ECD, and conventional LC, which are either non-specific, lack sensitivity, or require prior oxidation of the drug. HPLC methods are sensitive and rapid.

Pietta et al. [225] extracted plasma containing nifedipine and the internal standard (4-dimethylaminobenzaldehyde) into EtOAc. The organic layer was evaporated, and the residue was dissolved in MeOH for injection. Chromatographic conditions were: column = μBondapak C_{18}, mobile phase = 55:45, MeOH:0.01 M Na_2HPO_4, pH 7.2, flow-rate = 2 mL/min, and detector = 254 nm. Nifedipine (t_R = 7.5 min) and metabolites were separated by this procedure. Dokladalova et al. [226] used a 5-μm Nucleosil C_{18} column, mobile phase (H_2O:CH_3OH:CH_3CN, 55:36:9) pumped at 1 mL/min, detector set at 235 nm. Nifedipine eluted at 15 min. A simple, selective, and sensitive method was

developed by Bratin and Kissinger [227] for nifedipine, its decomposition products, and its metabolites. Alkalinized plasma was extracted with EtOAc. After evaporating the organic layer, the residue was dissolved in mobile phase, and 50 μL of the solution was injected (without removal of oxygen) on a 5-μm Biophase ODS column. The detector consisted of dual electrodes: either two thin-layer glassy carbon electrodes or one glassy carbon and one gold electrode. The mobile phase consisted of 0.02 M monochloroacetic acid, 0.015 M NaOAc, 0.001 M Na_2EDTA in 20% (v/v) *n*-propanol, and 5% (v/v) EtOH. The retention times (at a flow-rate of 1.2 mL/min) for two photodecomposition products and nifedipine were 8, 11, and 12 min, respectively. Detection limit was about 20 ng/mL of plasma.

2. *Verapamil*

Verapamil, a slow channel-blocking agent, is well absorbed (>90%) when given orally, but its bioavailability is low (13-35%). Plasma levels peak in 1-2 hr and decline with a $t_{\frac{1}{2}}$ of 1.6-7.0 hr [228-231]. Plasma $t_{\frac{1}{2}}$ is increased several-fold in patients with liver cirrhosis [232]. Verapamil is rapidly and extensively metabolized to at least 12 metabolites: the major active one, norverapamil, has a $t_{\frac{1}{2}}$ of 3.6-8.0 hr. Repeated oral administration of verapamil results in decreased clearance, and accumulation of the drug and norverapamil [228-231, 233, 234].

A sensitive and specific HPLC assay developed by Harapat and Kates [235, 236] has been used by several investigators [228,229,233, 234] to quantitate verapamil and norverapamil in blood and urine. The sample, with added internal standard (an analogue of verapamil), was alkalinized and then extracted with Et_2O. Et_2O layer was reextracted with dil. H_2SO_4. The aliquot was analyzed by a reversed-phase column using fluorescence detection. Jaouni et al. [237] used a μBondapack C_{18} column, CH_3CN:0.004 N H_2SO_4, pH 2.4 (29:71) as the mobile phase and flow-rate of 2.5 mL/min. Verapamil, norverapamil, and two other metabolites eluted at 13.0, 11.7, 3.5, and 3.2 min, respectively. Cole et al. [238] used methyl *t*-butyl ether to extract alkalinized plasma containing 5,6-benzoquinoline as the internal standard. The chromatographic parameters were: column = 5-μm Spherisorb 5 silica, mobile phase = methanolic solution of KBr (3mM) and $HClO_4$ (0.37 mM), flow-rate = 2 mL/min, and fluorescence detection. Verapamil, norverapamil, and the internal standard eluted at 4.0, 2.0, and 5.5 min, respectively. The sensitivity was 2 ng/mL. Kuwada et al. [239] extracted plasma containing internal standard with Et_2O. After evaporating the Et_2O, the residue was dissolved in mobile phase. Chromatographic conditions were: a reversed-phase column (30°C); mobile phase = 1:1 mixture of CH_3CN and an aqueous solution of NaH_2PO_4 (0.71 g), H_3PO_4 (0.3 mL), 1-heptanesulfonic acid (2 g) in 1 liter water and adjusted to pH 4.6; flow-rate = 1.5 mL/min; and fluorescence detection.

3. *Diltiazem*

About 80% of an oral dose is absorbed. Bioavailability of diltiazem varies widely (27-74%), due to interindividual differences in the first pass effect. Plasma levels peak in 2-3 hr. After i.v. administration, plasma levels of the drug decline biexponentially: $t_{\frac{1}{2}}\alpha = 0.3 \pm 0.2$ hr, $t_{\frac{1}{2}}\beta = 3.1 \pm 1.0$ hr. After an oral dose, $t_{\frac{1}{2}}\beta$ is in the range of 4-6 hr [240-242]. Diltiazem undergoes extensive hepatic biotransformation with only 2-4% of the dose appearing in urine as unchanged drug. Severe renal failure does not alter the pharmacokinetic parameters of the drug or the metabolite [241].

Diltiazem and desacetyldiltiazem have been measured by GC [243, 244], and by HPLC [242,245]. Verghese et al. [245] extracted plasma containing the internal standard (verapamil) with methyl *t*-butyl ether. The organic phase was back-extracted with dil. H_2SO_4. Aliquots were injected on a reversed-phase 6 μm Zorbax CN column. Mobile phase was 45:55:9.25 v/v mixture of MeOH: 0.05 M NH_4HPO_4: triethylamine, adjusted to pH 5.0. Flow-rate was 1.5 mL/min. Detection was 237 nm. Desacetyldiltiazem, diltiazem, and verapamil eluted at 2.5, 3.35, and 4.0 min, respectively. The sensitivity was 10 ng/mL in plasma.

IX. CONCLUSION

Sensitive and specific HPLC methods are now available for the measurement of almost all of the presently available antihypertensive drugs and their important metabolites. In most cases the HPLC procedures are rapid and less cumbersome than alternate modes of analysis.

Determination of plasma levels of antihypertensive agents may not be of value since (a) large interindividual differences are noted in plasma levels resulting from the same dose of an antihypertensive drug, and (b) large variations have been observed in plasma drug concentrations that produce the same therapeutic response in different patients. However, if a relationship has been established between the therapeutic effect and the plasma level of a drug, for an individual patient, plasma level measurement might be helpful in defining the optimum dose of the drug for that patient. Plasma level monitoring of certain drugs and active metabolites may be important in patients with hepatic and/or renal impairment, with propensity towards adverse side effects.

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14

ANTIARRHYTHMICS

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I. INTRODUCTION

The antiarrhythmic agents procainamide, *N*-acetylprocainamide (NAPA), lidocaine, quinidine, disopyramide, and propranolol are important in the treatment of a variety of cardiac disorders. Antiarrhythmic drugs can selectively suppress the automaticity at ectopic sites. Quinidine was first used for its antiarrhythmic effect in 1914 [1], and is still considered the prototype among this group. Quinidine depresses the excitability of both the atrium and the ventricle to electrical stimulation. Automaticity of pacemaker cells is decreased and the conduction velocity of cardiac impulse is slowed. In 1936, the antifibrillatory and antiarrhythmic effects of procaine were reported [2]. However, this agent was clinically unsuitable because of its central nervous system stimulation and its degradation by enzymatic hydrolysis. On the other hand, procainamide, an amide derivative of procaine, is an effective antiarrhythmic drug which has negligible central nervous system effects and is stable to enzymatic hydrolysis. The pharmacological activity of procainamide is very similar to that of quinidine except for a difference in their potencies.

Lidocaine is used as a local anesthetic [3]. It is also an effective antiarrhythmic agent primarily used for ventricular arrhythmias. Propranolol, a β-adrenergic blocking agent, is sometimes also used as an antiarrhythmic agent [14]. Propranolol reduces contractility. It is specially useful in treating ventricular arrhythmias induced by digitalis intoxication. Disopyramide, a new antiarrhythmic agent [5], was introduced in the United States in 1977. It is similar to quinidine and procainamide in its antiarrhythmic activity.

A. Pharmacokinetics of Antiarrhythmic Drugs

For most drugs the relation between dosage and intensity of the pharmacological effect is unpredictable. An interpatient variation in the dose-effect relationship may arise largely from genetically determined individual differences in drug absorption, distribution, biotransformation, and excretion. Other factors such as age, sex, effects of disease, and concomitant administration of other drugs also contribute to this variation. For a number of drugs, the correlation between serum concentrations and clinical efficacy has been established. Determination of the serum concentration of these drugs can help in the individualization of drug dosage to achieve optimum therapeutic efficacy without adverse toxicity.

The antiarrhythmic drugs described above have narrow therapeutic ranges and adverse side effects. These agents show good correlation between their serum concentrations and clinical effects. Therefore, it is usually desirable to monitor serum concentrations of these drugs to serve as therapeutic guides. The therapeutic blood concentrations, dosages, and pharmacokinetic parameters of these agents are illustrated in Table 1.

B. Metabolism and Biotransformation

Procainamide is almost completely absorbed after oral administration and is rapidly distributed in body tissues [11]. Peak plasma concentrations are generally reached within 1–2 hr, and about 15% of procainamide binds to plasma proteins. Procainamide is eliminated mainly via the kidney, and 50% is excreted unchanged in the urine. *N*-Acetylprocainamide (NAPA) is the major metabolite formed by the liver and accounts for 15% of the urinary excretion of administered procainamide. The rate of acetylation is genetically determined [12]. NAPA is believed to have comparable toxicity as well as therapeutic potency to procainamide [13]. Its concentration in plasma ranges from 60 to 180% of procainamide. Therefore, it is important to measure both procainamide and NAPA concentrations as a guide to therapy.

Quinidine is fully absorbed from the gastrointestinal tract, but the fraction of the oral dose which reaches the systemic circulation is approximately 75%. This reduction in bioavailability is due to first-pass hepatic drug removal [14]. Quinidine is bound to plasma proteins to the extent of 70–95%. Fifteen to 40% of the quinidine is eliminated by renal excretion as intact drug, and 60–80% as various metabolites [7]. At least four different metabolites of quinidine have been identified in plasma, including 3-hydroxyquinidine, 2'-quinidinone, O-desmethylquinidine, and quinidine-*N*-oxide. The clinical effect is mediated not only by parent compound but also by metabolites. However, the relative contribution of each metabolite to an observed effect remains to be established [15].

Table 1 Properties of the Antiarrhythmic Drugs

Drug	Use	Serum half-life (hr)	Route	Dose (mg/kg/D)	Therapeutic range (mg/L)	Toxic level (mg/L)	Volume of distribution (L/kg)
Procainamide	Prevention of premature ventricular beats, ventricular tachycardia	2–4	Oral i.v.	50	4–8	>16	2
Metabolite: *N*-Acetyl-procainamide		6–8		Serum concentration ranges from 60 to 180% of procainamide.			
Quinidine	Treatment of atrial fibrillation	5–12	Oral	10–20	2–5	>10	2.0–3.5
Lidocaine	Treatment of ventricular tachycardia (acute myocardial infarction)	1–2	i.v. i.m.	30–40	1–5	>6	1.7
Propranolol	Treatment of digitalis arrhythmias	2–6	Oral	1.1–1.7	50–100 μg/L	variable	2–4.6
Disopyramide	Treatment of ventricular tachycardia	4–10	Oral i.v.	8.6	2–4	>7	0.8
Metabolite: *N*-Desisopropyldisopyramide							

Source: Refs. 6–10.

The oral bioavailability of lidocaine is about 35%. Lidocaine is metabolized predominantly by the liver. Excretion of unchanged lidocaine is a minor route of elimination. Two deethylated metabolites of lidocaine, monoethylglycylxylidide (MEGX) and glycylylidide (GX), are found in significant concentrations in the blood of patients receiving lidocaine therapy [16]. MEGX and GX have been shown to have antiarrhythmic activities of 83% and 10–26%, respectively [17]. Accumulation of metabolites during prolonged intravenous administration may account for the observed toxicity which occurs despite blood lidocaine concentrations which are within the therapeutic range.

Propranolol is completely absorbed after oral administration. Peak plasma concentrations are achieved in approximately 2 hr [18]. The low bioavailability (about 35%) is due to avid first-pass hepatic extraction. After intravenous administration, most of the propranolol is excreted in the urine as metabolites which are formed in the liver. Four primary metabolic pathways have been discovered: O-dealkylation, side-chain oxidation, glucuronic acid conjugation, and ring oxidation. The inactive propranolol glucuronide accounts for 75% of the urinary excretion. 4-Hydroxypropranolol, an active metabolite, is observed only after oral administration [19].

The bioavailability of orally administered disopyramide phosphate is about 90% but it is absorbed much more slowly. Peak serum levels are generally achieved between 2 and 3 hr after administration [20]. Disopyramide is excreted unchanged up to about 55% of the dose in urine; about 25% appears in the urine as *N*-desisopropyldisopyramide; and 10% as minor metabolites. *N*-Desisopropyldisopyramide may be 25% as potent as the parent drug [21].

C. History of Analytical Methods

A variety of analytical techniques have been employed for the analysis of antiarrhythmic drugs. Many of the initial studies on the therapeutic or toxic effects of these drugs were facilitated by the use of colorimetric [22,23], fluorometric [24–28], and spectrophotometric methods [29]. Some of the problems associated with these techniques are the requirement of a large amount of sample and the potential interference by other closely related compounds. Fluorometric techniques have been extensively used for the analysis of procainamide, NAPA, quinidine, propranolol, and disopyramide. The interference caused by metabolites have severely limited the usefulness of this technique for therapeutic drug monitoring. In spite of these limitations, several fluorometric methods are still used for the analysis of quinidine in clinical laboratories [27,28].

Thin-layer chromatography possesses adequate resolution for identifying many of the antiarrhythmic agents but suffers from the inability

to accurately and precisely quantitate these drugs. It is also laborious and time consuming; nevertheless, it remains a powerful technique in the toxicology laboratory for the identification of these drugs [30,31].

Several gas-liquid chromatographic (GLC) methods were reported for the analysis of antiarrhythmic drugs [32]. Different derivatization procedures, columns, and detectors were used to analyze various drugs. The adsorption of antiarrhythmic drugs on the active sites of packing material caused nonlinear response at low concentrations.

Enzyme multiplied immunoassay techniques (EMIT) have been successfully applied for the analysis of antiarrhythmic drugs [33]. This technique is sensitive, simple, and rapid. However, the high cost of reagents, the need to assay parent drug and metabolites separately, and the cross-reactivity of antibodies with closely related metabolites have limited the utility of this technique in the pharmacokinetic studies.

Liquid chromatography offers several advantages over these techniques. Sample manipulation and derivatization prior to chromatography is minimal, and several classes of compounds, including their metabolites, can be assayed simultaneously with good precision, specificity, and accuracy. Several liquid chromatographic methods for the simultaneous analysis of antiarrhythmic drugs have been reported. Lagerstrom et al. [34] developed an LC method for the analysis of disopyramide, lidocaine, procainamide, and quinidine using liquid-solid adsorption chromatography. A single sample-preparation technique but different analytical parameters, including column packing size, mobile phase, and detection wavelengths, limited the utility of this method. A simultaneous reversed-phase method for the analysis of disopyramide, lidocaine, and quinidine was reported by Flood et al. [35]. After addition of *p*-chlorodisopyramide as internal standard, serum was extracted at a basic pH, chromatographed on a reversed-phase C_{18} column, and detected at 254 nm. We developed a reversed-phase method for the simultaneous analysis of the antiarrhythmic drugs procainamide, NAPA, lidocaine, quinidine, disopyramide, *N*-desisopropyldisopyramide, and propranolol by gradient elution [36]. For laboratories without gradient capabilities, two simple isocratic methods which differ only in the composition of their mobile phases were reported. This method offers several advantages over other existing methods. It provides for simultaneous analysis of five drugs and two metabolites. The sensitivity (10 μg/L for propranolol with UV detection, 1.0 μg/L with fluorescence detection, and 0.1 mg/L for all other drugs) allows for the measurement of both the parent drug and its metabolites at low concentrations. The linearity of the assay covers both the therapeutic and toxic levels of each drug. Good recoveries and excellent precision with a simple, one step-extraction procedure offers simplicity, accuracy, and versatility for routine application in a clinical laboratory.

II. PREPARATION OF SAMPLES

The measurement of antiarrhythmic drugs is commonly performed on serum or plasma samples. The sample size varies between 50 μL and 1 mL, depending upon the sensitivity of the particular method being used. Methods utilizing fluorescence detection usually require less sample than the methods utilizing UV detection.

Current methods used to routinely monitor antiarrhythmic drugs do not discriminate between bound and free drug. It is generally recognized that only the free drug diffuses into tissues and interacts with an effector site to produce a response. The extent of protein binding varies greatly among different drugs, and there is a great difference in the degree of protein binding for the same drug within the population. In acute-phase reactions, many globulins, including α_1-acid glycoprotein, are elevated in plasma. This glycoprotein is the major plasma binding protein for propranolol, quinidine, and several other basic drugs; and correspondingly, the plasma binding and total plasma concentration of these drugs are elevated. In the therapeutic range, the binding of disopyramide may change with drug concentration [37]. It would therefore seem more reasonable to correlate free drug concentration with clinical efficacy or toxicity. Unfortunately, the techniques employed for the measurement of free drug are laborious and time consuming and require specialized apparatus, rendering them impractical for routine use.

Extraction methods for the isolation and concentration of antiarrhythmic agents from the specimen may vary from a simple one-step solvent extraction, to complicated back-extraction in aqueous acid. Generally the type of extraction and amount of sample cleanup is dictated by the efficiency and the selectivity of the chromatographic technique used for analysis. The more specific and efficient the chromatographic system, the less sample extraction and cleanup are necessary to obtain the desired results. Sometimes extraction steps are necessary to improve the sensitivity of the assay method by concentrating the analyte.

In the various liquid-liquid extraction methods employed for the analysis, typically 0.1–3 mL of serum is extracted with nonpolar organic solvents (methylene chloride, chloroform, ethyl acetate, etc.) at a basic pH. After phase separation, the organic layer is evaporated and the residue reconstituted into a small aliquot of mobile phase or polar solvents. The liquid-liquid extraction method eliminates most of the plasma proteins and acidic compounds. Excellent recoveries were reported for several antiarrhythmic drugs using this extraction procedure.

The liquid-liquid extraction procedures, in which serum or plasma is extracted with organic solvent followed by extraction of organic solvent into a small volume of aqueous acid, provide a much cleaner sample because acidic and neutral compounds from both endogenous and exogenous sources are excluded from the extracted residue. Additionally, the dilute acid into which the drugs and metabolites of interest are ex-

tracted is a suitable solvent for direct injection onto the reversed-phase columns. Finally adequate concentration of the sample occurs by back extraction into a small volume of dilute acid, so that time-consuming evaporation steps are not necessary. This back-extraction technique was utilized by Gotelli et al. [38] to analyze procainamide, NAPA, quinidine, and propranolol from serum. They extracted 100 μL of serum into 1 mL of methylene chloride, then back-extracted in 100 μL of 0.1% phosphoric acid. An aliquot of this phosphoric acid was directly injected into the LC.

A charcoal adsorption method was used by Adams et al. [39] to isolate procainamide and lidocaine from serum. The adsorbed drugs were eluted form the charcoal surface by methylene chloride. Although the reported precision and sensitivity of the assay was adequate, it suffered from poor recoveries.

In recent years numerous sample preparation techniques using a liquid-solid extraction principle have been introduced. Examples of products employed for solid phases are charcoal, XAD resins (Rohm & Haas, Philadelphia, Pennsylvania), and cartridges of bonded silica gels such as Sep-Pak (Waters Associates, Milford, Massachusetts), and Bond-Elut (Analytichem International). The solid surfaces can provide a specific interaction with the compound of interest which is quite different from the solubility mechanism of liquid-liquid partitioning. Solid phases also provide a large surface area on which these interactions can take place. Liquid-solid systems thus provide a separation mechanism which is more selective and more efficient than that associated with liquid-liquid partitioning [40].

Disposable extraction columns such as Clin-Elut or Bond-Elut can be used to extract a number of antiarrhythmic agents from serum or plasma. The Clin-Elute extraction method is based upon the principle of liquid-liquid extraction. The sample is introduced onto the top of the column that has been prepacked with an inert matrix of large surface area. A suitable organic solvent is used to elute the drugs, while the polar components, particulate matter, and other impurities are retained on the column. These columns are very versatile and easy to use for the extraction of several classes of drugs and endogenous compounds.

The Bond-Elut columns facilitated rapid extraction of antiarrhythmic drugs from serum or plasma samples with very high efficiency and precision. With use of Vac-Elute chamber, designed to accept 10 Bond-Elut columns simultaneously, we were able to process 10 serum samples in <5 min. These columns were used to extract quinidine, disopyramide, and lidocaine from serum samples for very-high-speed liquid chromatography [41]. The Bond-Elut columns could be regenerated for repetitive use by passing two column volumes of methanol through them; 10–15 serum samples could be processed without any appreciable loss in the extraction efficiency.

Protein precipitation is probably the simplest and most rapid method of sample preparation prior to LC analysis. The basic principle of this technique is to precipitate serum proteins in the sample using a water-miscible organic solvent such as acetonitrile, methanol, or acids such as trichloroacetic acid or perchloric acid. Although the proteins are precipitated, the drugs remain freely solubilized in the supernatant. Acetonitrile is probably the best solvent for this purpose because it yields a clear supernatant in a short period of time. However, sample dilution may be a problem for therapeutic agents which are present at low concentrations, such as tricyclic antidepressants, propranolol, and certain benzodiazepines. In addition to the problem of sample dilution, protein precipitation is not selective, since other compounds present in the sample will be injected into LC with the antiarrhythmic drugs and may interfere with the assay. This technique was successfully applied to monitor quinidine concentration in serum samples, using both UV and fluorescence detection [42,43].

III. CHROMATOGRAPHY

Since normal-phase columns (packed with silica) were the first to be introduced, many of the early investigators utilized these columns for the analysis of antiarrhythmic agents. Silica is a polar packing material possessing active hydroxyl groups on its surface. Because of the polar nature, it generally retards polar compounds, while the nonpolar compounds are eluted with ease. Since most of the antiarrhythmic drugs are basic in nature, they are strongly retarded by the acidic hydroxyl groups lying on the surface of silica. This interaction causes a severe tailing of the chromatographic peaks. Sometimes acids or bases are added to the mobile phase to favorably effect possible secondary equilibria, such as ion-pair formation or ion suppression. Normal-phase methods require more extensive sample cleanup prior to chromatography than reversed-phase methods, because the aqueous samples are not compatible with silica packing. Usually the polar metabolites are strongly retarded on the column and may interfere with the subsequent analysis.

A normal-phase chromatographic method was developed by Dutcher and Strong [44] for simultaneous analysis of procainamide and NAPA. After addition of *p*-nitro-*N*-(2-diethylaminoethyl)benzamide hydrochloride as an internal standard, serum was extracted with ethyl acetate, separated on a microparticulate silica column, and detected at 254 nm.

Lagerstrom et al. [34] reported a method for the analysis of disopyramide, lidocaine, procainamide, and quinidine using a silica column. A single, simplified sample preparation technique but different analytical parameters (including column packing size, mobile phase, and detection wavelength) limited its utility for routine application.

Peat and Jennison [45] separated quinidine and dihydroquinidine on a microparticulate silica column after extracting the serum sample with

ether. The incomplete resolution between quinidine and the internal standard made this procedure an unreliable method for quinidine quantitation. Another method utilizing ion-pair extraction and silica gel chromatography was reported by Sved et al. [46]. The laborious sample treatment and external standardization resulted in poor precision.

Among all the factors contributing to the surge of interest in therapeutic drug monitoring by LC, none stands out as prominently as the development of reversed-phase chromatography using alkyl chemically bonded phases. Bonded phases are typically made by reacting the appropriate chloro- or alkoxysilane with a fully hydroxylated silica gel. In reversed-phase chromatography, the stationary phase is less polar than the mobile phase. Analytes thus elute in a general order of decreasing polarity. Mobile-phase strength increases with decreasing polarity.

There are a number of favorable aspects of reversed-phase chromatography in therapeutic drug monitoring, the most important being the aqueous nature of the mobile phase, which is compatible with biofluids. The ability to separate a wide range of drugs and metabolites of varying hydrophobicities is particularly useful in the analysis of a complex mixture of compounds found in physiological fluids.

A number of reversed-phase LC methods have been reported for the analysis of antiarrhythmic drugs and their metabolites (Table 2). Usually, C_{18} bonded-phase packing and acetonitrile and water mobile-phase eluents are used to effect separations. Most of these reported methods utilize a simple liquid-liquid extraction procedure to isolate these drugs from serum matrix at alkaline pH. The commonly used solvents for extraction are methylene chloride, diethylether, and ethyl acetate. Adams et al. [39] reported a reversed-phase method for the simultaneous analysis of lidocaine and procainamide using procaine as an internal standard. Although charcoal extraction yielded poor recoveries, detection at 206 nm overcame the problem of insensitivity. Poor recoveries and interference of NAPA with procaine prompted them to modify their procedure to incorporate a liquid-liquid extraction procedure with carbocaine as an internal standard [47]. Carr et al. [48] and Rocco et al. [49] reported similar reversed-phase methods for the analysis of procainamide and NAPA. They isolated the drugs using a simple liquid-liquid extraction method, and then detected them at 254 or 280 nm. Shukur et al. [50] developed a reversed-phase LC method for procainamide and NAPA without using any internal standard. Analytical recoveries of 55% and external standardization resulted in poor precision.

Reversed-phase is ideally suited for the determination of quinidine and many of its polar metabolites from physiological fluids. Most of the reported reversed-phase methods employed a liquid-liquid extraction technique to isolate quinidine and its metabolites from biofluids. Powers and Sadee [42] developed a simple and rapid reversed-phase

Table 2 Review of LC Procedures That Can Monitor Antiarrhythmic Drugs at Therapeutic Concentrations

Drug	Sample preparation	Column[a]	Detection (nm)	Remarks	Reference
Procainamide + NAPA	Charcoal adsorption 1.0 mL serum	C_{18} RP	205	Procaine (I.S.) NAPA not analyzed	39
	0.5 mL serum extracted with 10% *n*-propanol in chloroform	C_{18} RP	254	*N*-Formylprocainamide (I.S.)	48
	0.2 mL serum extracted with diethyl ether	C_{18} RP	280	External standardization	50
	0.5 mL serum extracted with methylene chloride	C_{18} RP	280	*N*-Propionylprocainamide (I.S.)	49
	1.0 mL serum extracted with ethyl acetate	SG	254	*p*-Nitro-*N*-(2-diethylaminoethyl)benzamide (I.S.)	44
	0.1 mL plasma + 0.25 mL acetonitrile	Partisil SCX Cation exchange	274	External standardization	63
	2 mL plasma extracted with methylene chloride	SG	254	Pheniramine maleate (I.S.)	64
	0.5 mL serum extracted with methylene chloride	C_8 RP	280	*N*-Propionyl procainamide (I.S.)	36
	0.1 mL serum extracted with methylene chloride and back-extracted in 0.1% phosphoric acid	C_8 RP	280	*N*-Propionylprocainamide (I.S.)	65

	0.2 mL serum extracted with methylene chloride	SG	280	External standardization	34
Quinidine	1 mL plasma extracted with ether	SG	280	1-Cinchonidine (I.S.)	45
	0.5 mL plasma + 0.25 mL perchloric acid + methyl isobutyl ketone	SG	Fluorescence excitation, 325 Emission filter, 420	Counter-ion formation with perchloric acid	46
	0.5 mL serum extracted with benzene	C_{18} RP	254	Theobromine (I.S.) added after extraction	66
	0.2 mL serum + 0.2 mL acetonitrile	RP-alkylphenyl	330	External standardization	42
	1 mL plasma extracted with benzene	Partisil SCX cation exchange	230	Cinchonine (I.S.)	67
	0.05 mL serum extracted with benzene	C_{18} RP	Flurorescence excitation, 340 Emission filter, kV 418	1-Cinchonidine (I.S.) Separation of metabolites	68
	0.1 mL serum extracted with toluene	C_{18} RP	UV254, and Fluorescence excitation, 340 Emission, 430-640	Quinine (I.S.)	69
	2 mL plasma extracted with ether:methylene chloride:isopropanol)	SG	235	Primaquine (I.S.) (Separation of metabolites)	56

Table 2 (Continued)

Drug	Sample preparation	Column[a]	Detection (nm)	Remarks	Reference
	1 mL urine extracted with methylene chloride:iso-propanol	RP-alkylphenyl	230	Oxyprenolol (I.S.) (Separation of meta-bolites)	70
	0.2 mL plasma extracted with methylene chloride: isopropanol	RP-alkylphenyl	Fluorescence excitation, 245 Emission, 340	Pronethalol (I.S.) Separation of meta-bolites	15
	0.2–0.5 mL plasma + twice the volume of acetonitrile	C_8 RP	Fluorescence excitation, 245 Emission, 435	3-Methyl-5-triazolo-phthalazine (I.S.) Separation of meta-bolites	43
	0.2–0.5 mL serum extrac-ted with methylene choloride or ether	C_8 RP	UV, 216; and Fluorescence excitation, 290 Emission, 350	Pronethalol (I.S) Separation of meta-bolites	36
	0.1 mL serum extracted with isopropanol:methylene chloride, back-extracted in 0.1% phosphoric acid	C_8 RP	Fluorescence excitation, 290 Emission, 350	Pronethalol (I.S.)	38
	0.5 mL serum extracted with methylene chloride	C_{18} RP	254 or 205	*p*-Chlorodisopyramide (I.S.)	35
Lidocaine	Charcoal adsorption of 1 mL serum	C_{18} RP	205	Procaine (I.S.)	39

	0.5 mL plasma extracted with ethyl acetate	RP-alkylphenyl	200	Ethylmethylglycine-xylidide (I.S.) Separation of metabolites	55
	Direct injection of serum through guard column	C_{18} RP	205	External standardization Separation of metabolites	51
	0.5 mL serum extracted with methylene chloride	C_{18} RP	205	*p*-Chlorodisopyramide (I.S.)	35
	0.5 mL serum extracted with methylene chloride	C_8 RP	216	*p*-Chlorodisopyramide (I.S.) Separation of metabolites	36
	0.5 mL serum extracted with chloroform:hexane: isopropanol	C_{18} RP	210	Ethylmethylglycylxylidide (I.S.) Separation of metabolites	54
	0.2 mL serum extracted with methylene chloride	SG	228	External standardization	34
Diso-pyramide	0.1—1.0 mL of plasma or urine extracted with ether, back-extracted in 0.1 N sulfuric acid	C_{18} RP	254	*p*-Chlorodisopyramide (I.S.) Heptanesulfonic acid as ion-pair reagent	71
	1 mL plasma extracted with methylene chloride	C_{18} RP	258	*p*-Chlorodisopyramide (I.S.)	72
	1 mL plasma extracted with chloroform	Cyano	254	*p*-Chlorodisopyramide (I.S.)	73
	0.5 mL plasma extracted with chloroform	C_{18} RP	254	*p*-Chlorodisopyramide (I.S.)	74

Table 2 (Continued)

Drug	Sample preparation	Column[a]	Detection (nm)	Remarks	Reference
	0.5 mL serum extracted with methylene chloride	C_8 RP	216	*p*-Chlorodisopyramide (I.S.)	36
	0.5 mL serum extracted with methylene chloride	C_{18} RP	205 or 254	*p*-Chlorodisopyramide (I.S.)	35
Propranolol	2 mL plasma extracted with ether	C_{18} RP	Fluorescence excitation, 295 Emission, 360	Pronethalol (I.S.) Heptane sulfuric acid counter-ion	59
	2 mL plasma extracted with hexane:isoamyl alcohol	C_{18} RP	Fluorescence excitation, 285 Emission, 350	Pronethalol (I.S.)	52
	1 mL serum extracted with cyclohexane	Cyano	Fluorescence excitation, 210 Emission, 340 Cut-off filter	Pronethalol (I.S.)	75

1 mL plasma extracted with butanol in hexane	Cyano	Fluorescence excitation, 220 Emission, no filter	External standardization 4-hydroxypropranolol analyzed at different fluorescence setting	76
1 mL plasma extracted with ethylacetate and back-extracted with dilute sulfuric acid	RP-alkylphenyl	Fluorescence excitation, 205 Emission, kV 340 filter	4-Methyl propranolol (I.S.) Analysis of metabolite	58
1 mL plasma extracted with methylene chloride	C_8 RP	UV, 216 or Fluorescence excitation, 290 Emission, 350	*p*-Chlorodisopyramide (I.S.) Pronethalol (I.S.)	36
200 μL plasma extracted with methylene chloride and back-extracted in 0.1% phosphoric acid	C_8 RP	Fluorescence excitation, 290 Emission, 350	Pronethalol (I.S.)	53

[a]RP, Reversed phase; SG, silica gel.

method for the analysis of quinidine. They precipitated the serum proteins by adding equal volume of acetonitrile, and then injected the serum supernatant. Gotelli and Wall [38] back-extracted the quinidine and its metabolites from methylene chloride into 0.1% phosphoric acid, and injected an aliquot of the acid phase into the liquid chromatograph.

Lidocaine and its metabolites were extracted from serum at alkaline pH and analyzed on a C_{18} column (Table 2). Direct injection of serum samples through a precolumn onto a C_{18} analytical column was used by Wisnicki et al. [51] for the analysis of lidocaine and its metabolites. The precolumn had to be replaced after every 15 analyses to protect the analytical column.

Several reversed-phase LC methods have been reported for the analysis of disopyramide and its dealkylated metabolite (Table 2). Most of these methods employ a simple liquid-liquid extraction procedure followed by UV detection.

Reversed-phase methods for the analysis of propranolol frequently utilize fluorescence detection because of the low therapeutic concentration of this drug. Typically, 1–2 mL of plasma is extracted at alkaline pH with nonpolar organic solvent. After evaporating the organic solvent, the residue is reconstituted in the polar mobile phase and injected onto liquid chromatograph. Jatlow et al. [52] and Gotelli et al. [53] reported extraction methods incorporating back extraction of propranolol into phosphate buffer, which was directly injected into the chromatograph. These back-extraction procedures reduced the number of interfering substances observed in the chromatogram.

The diversity of antiarrhythmic drugs poses special problems for the simultaneous LC analysis of these agents: different columns are sometimes necessary; relatively large amounts of sample are required; and in general, different procedures are used for each individual drug. A simultaneous assay for disopyramide, lidocaine, and quinidine was reported by Flood et al. [35]. In our laboratory, a reversed-phase method for the simultaneous analysis of procainamide, NAPA, lidocaine, quinidine, disopyramide, *N*-desisopropyldisopyramide, and propranolol was developed using gradient liquid chromatography [36]. Two simple isocratic methods, which differ only in the composition of their mobile phases, were developed for laboratories without gradient capability (Figs. 1–5). Because of the great similarities between the two isocratic methods, the equilibration time necessary for switching between the two methods is less than 10 min. The simultaneous assay offers several distinct advantages over existing LC methods. The method eliminates the need for using different columns, detectors, and extraction procedures required for each analysis.

IV. DETECTION AND QUANTITATION

Virtually all LC methods for the analysis of antiarrhythmic drugs employ UV or fluorescence detection to monitor the column effluent. Many of

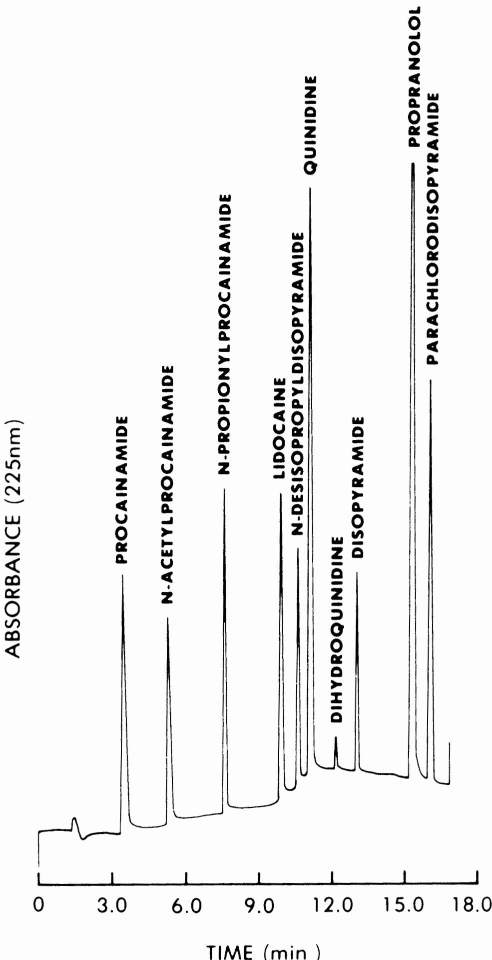

Figure 1 Chromatogram of a standard mixture of drugs run under gradient conditions. (Reproduced from Ref. 36, by courtesy of *Therapeutic Drug Monitoring.*)

these drugs can be detected at 254 nm if they are isolated and concentrated from biofluids prior to chromatography. However, lidocaine possesses very poor UV absorption at 254 nm and therefore cannot be detected in therapeutic concentration at this wavelength. But lidocaine exhibits a strong absorption shoulder near 205 nm, which could be used to monitor it in serum samples. Although the sensitivity is greatly enhanced at this wavelength, there are few limitations with the detection

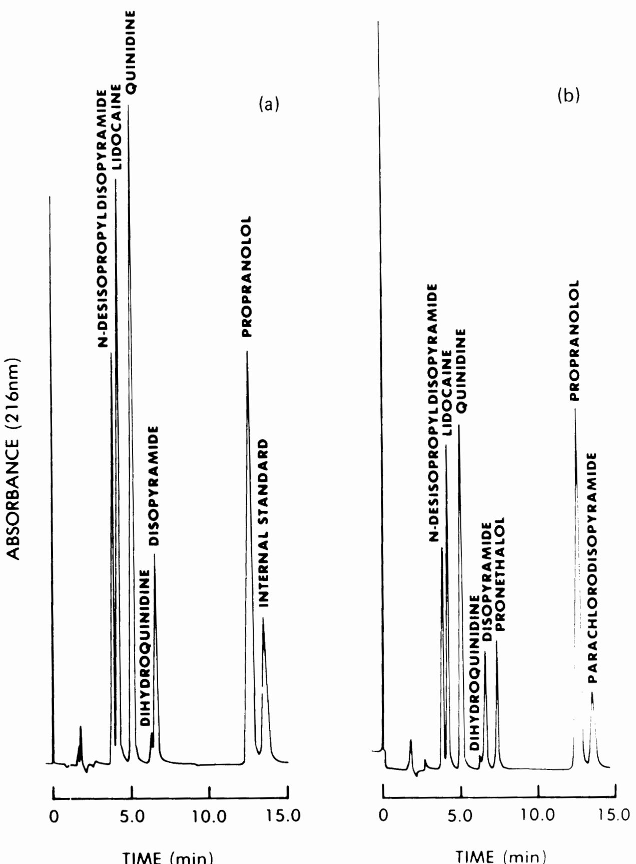

Figure 2 (a) Chromatogram of a standard mixture of antiarrhythmic drugs under isocratic elution with *p*-chlorodisopyramide as internal standard. (b) Chromatogram of the same standard with pronethalol as internal standard. (Reproduced from Ref. 36, by courtesty of *Therapeutic Drug Monitoring.*)

in this region. First, the mobile phase must be transparent at this wavelength. A mobile phase that usually conforms to these criteria of transparency is a mixture of phosphate buffer, acetonitrile, and methanol. Additionally, the detection wavelength is fairly nonspecific; hence, the resolution must be adequate between the analyte and potentially interfering substances. Procainamide, NAPA, quinidine, and disopyramide can be detected at either 254 nm or 280 nm by using a fixed-wavelength detector. Propranolol can be monitored at 216 nm.

Fluorescence is a powerful technique for detecting compounds that are present at low concentrations. This detection technique was suc-

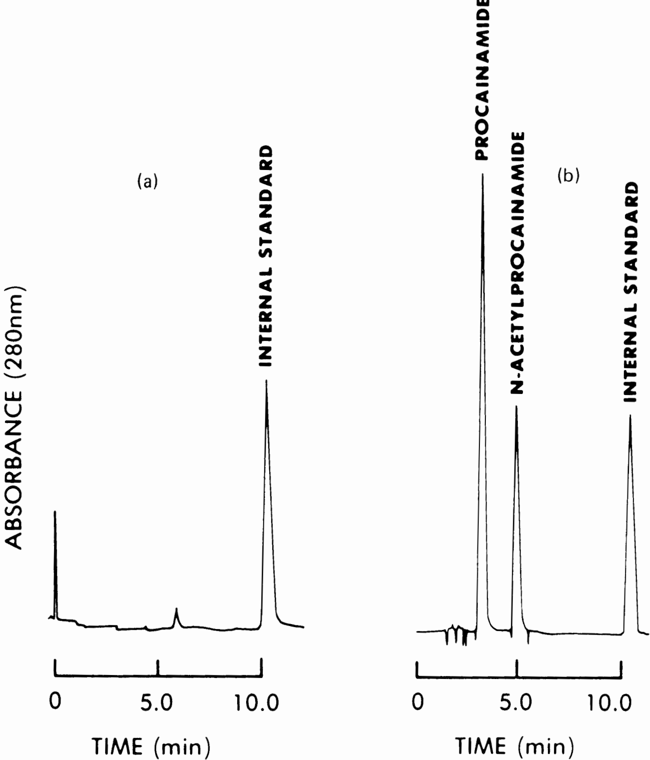

Figure 3 (a) Chromatogram of a drug-free serum with 10 mg of added internal standard (*N*-propionylprocainamide) per liter under isocratic elution. (b) Chromatogram from a patient's serum containing 9.9 mg/L of procainamide and 8.4 mg/L of NAPA. (Reproduced from Ref. 36, by courtesy of *Therapeutic Drug Monitoring*.)

cessfully utilized for the analysis of quinidine and propranolol. Both of these agents exhibit strong fluorescence and hence can be detected at nanogram concentrations. Also, fluorescence detection is more selective than UV detection, and usually endogenous constituents that are present in the physiological fluids will not interfere.

Quantitation can be accomplished either by peak height or peak area measurements, provided the chromatographic peaks are Gaussian or symmetrical. Internal standardization is the preferred quantitation method because it usually compensates for a number of variables in the assay.

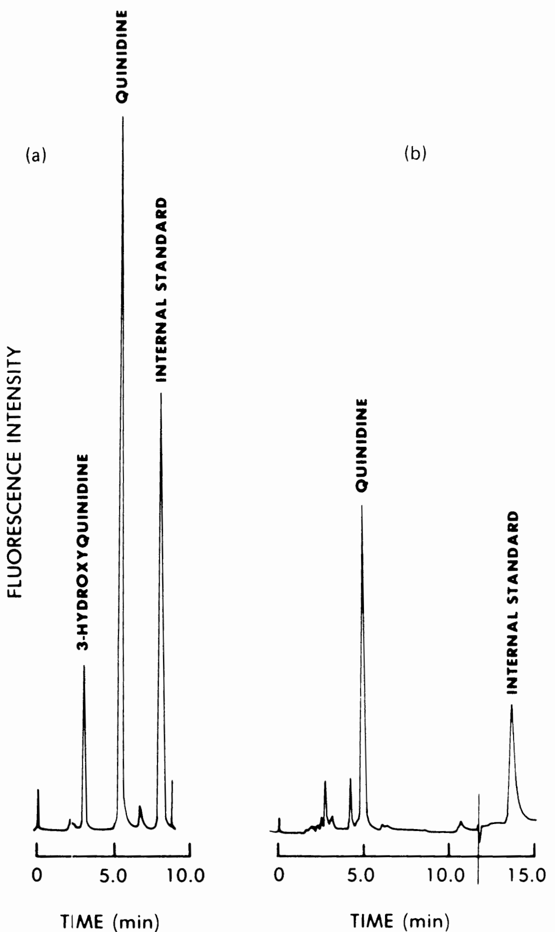

Figure 4 (a) Chromatogram from a patient's serum containing 5.0 mg/L of quinidine (fluorescence detection) under isocratic elution. (b) Chromatogram from a patient's serum containing 5.7 mg/L of quinidine (UV detection) under isocratic elution. (Reproduced from Ref. 36, by courtesy of *Therapeutic Drug Monitoring.*)

V. ANALYSIS OF METABOLITES

A number of antiarrhythmic drugs are transformed into bioactive metabolites. For instance, procainamide is metabolized into NAPA, which also possesses antiarrhythmic activity. Because NAPA has lower clearance than procainamide, it will accumulate to a greater degree. Steady-state levels of NAPA are considerably higher in rapid acetylators than

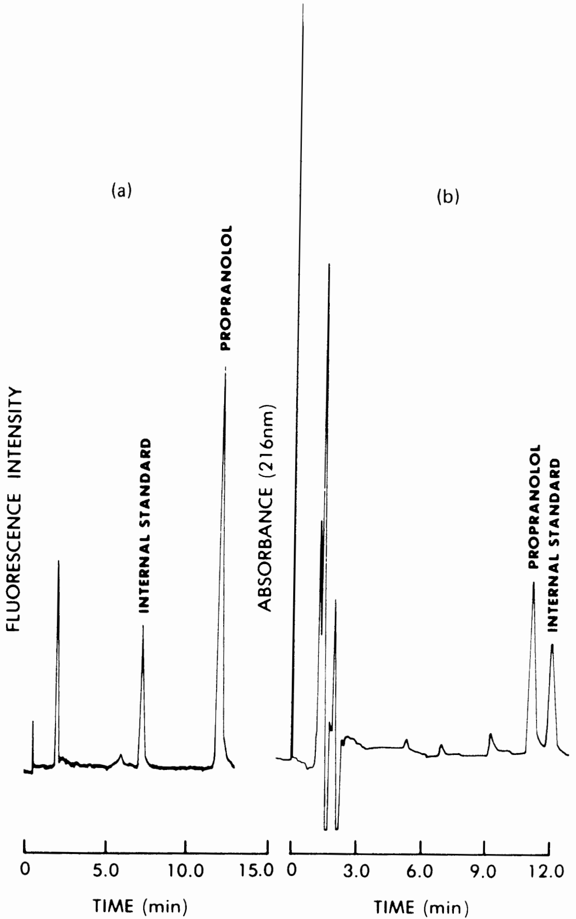

Figure 5 (a) Chromatogram from a patient's serum containing 100 μg/L of propranolol (fluorescence detection) under isocratic elution. (b) Chromatogram from a patient's serum containing 140 μg/L of propranolol (UV detection) under isocratic elution. (Reproduced from Ref. 36, by courtesy of *Therapeutic Drug Monitoring*.)

in slow acetylators. Accordingly, it is helpful to measure the parent drug and metabolite in plasma. Most of the LC methods discussed in this chapter are capable of analyzing them simultaneously.

The dealkylated metabolites of lidocaine, MEGX, and GX have been reported to be approximately 80 and 10%, as potent as the parent drug [17]. Furthermore, MEGX has been suggested as a major contributing factor in the occurrence of toxic reactions arising from the administra-

tion of lidocaine. Therefore, monitoring of plasma levels of lidocaine and MEGX may be beneficial in the control of antiarrhythmic therapy. Several reversed-phase methods have been reported for the analysis of lidocaine and its metabolites (Table 2). Narang and Crouthamel [54] analyzed lidocaine, MEGX, and GX by reversed-phase LC with UV detection at 210 nm. They extracted 0.5 mL of serum at alkaline pH with a solvent consisting of chloroform:hexane:isopropanol (60:30:10, v/v/v) and reported excellent recoveries for these compounds. Ethylmethylglycylxylidide (EMGX) was used as an internal standard. Urine samples can also be assayed after diluting them 10-fold with distilled water. Chromatography is complete in less than 10 min. Direct injection of serum samples through a precolumn onto an analytical C_{18} column was used by Wisnicki et al. [51] to measure lidocaine, MEGX, and GX. Without the usual concentration step, the method did not provide adequate sensitivity at low concentrations.

A reversed-phase method employing a μBondapak alkylphenyl column was developed by Nation et al. [55]: 500 μL of serum was extracted at alkaline pH with ethyl acetate, and back-extracted into 100 μL of dilute sulfuric acid. Approximately 20–30 μL of the aqueous acid phase was injected into the chromatograph. Lidocaine, MEGX, and GX were separated in about 10 min, and the column effluent was monitored at 200 nm. No interference from other antiarrhythmic drugs was observed.

A simultaneous assay of quinidine, dihydroquinidine, and four quinidine metabolites (2'-quinidinone, 3-hydroxyquinidine, O-desmethylquinidine, and quinidine-*N*-oxide) was reported by Guentert et al. [56]. Normal phase chromatography with detection at 235 nm provided a sensitivity of 20 μg/L when 2 mL of plasma was extracted. Quinidine and metabolites can be separated in less than 20 min with a mobile phase containing hexane:ethanol and ethanolamine. Quinidine and dihydroquinidine were only partially resolved under these conditions. Subsequently, they separated these compounds on a reversed-phase alkylphenyl column [15], and the effluent was monitored by fluorescence detection. A ternary mobile phase of phosphate buffer, acetonitrile, and tetrahydrofuran (pH 4.75) gave baseline separation between the parent drug and its metabolites. The chromatography was complete in about 35 min with a detection limit of about 15–20 ng/mL. The pH of the mobile phase was found to be crucial in the separation of all compounds. At low pH (2.5–3.5), quinidine-*N*-oxide was not separable from quinidine using various buffer systems and organic monifiers. At higher pH (5–7), the two compounds were resolved, but baseline separation of 2'-quinidinone and 3-hydroxyquinidine was not possible. Therefore, the intermediate pH (4.75) employed in this analysis represents a compromise for solving the two separation problems. Tetrahydrofuran is added in small amounts to the mobile phase because it eliminates tailing and promotes sharper peaks resulting in a better separation.

Rakhit et al. [43] separated quinidine and its metabolites in about 14 min. Plasma proteins were precipitated by adding two volumes of acetonitrile containing an internal standard. The quinidine and its metabolites were separated on an octyl reversed-phase column with a mobile phase consisting of acetonitrile:methanol:tetrahydrofuran and triethylamine. A pH of 2.5 was needed to separate quinidine-*N*-oxide from quinidine. The addition of tetrahydrofuran promoted sharper peaks, better separation, and decrease in total retention time.

Propranolol is mainly metabolized in liver. Many of the metabolites of propranolol have been identified in human urine. 4-Hydroxypropranolol was subsequently shown to have β-adrenergic blocking properties equivalent to those of propranolol [19]. Its measurement has been difficult because of the low concentrations present in serum and its short half-live. Analytically, the determination is further complicated by the fluorescent characteristics it possesses which are different from those of propranolol. Propranolol and its six metabolites were separated on a reversed-phase column by Pritchard et al. [57]. Propranolol and its neutral and basic metabolites were extracted into ether from urine at pH 9.8 and separated on a C_{18} column. The fluorescence intensity of the column effluent was measured by an Aminco fluoromonitor equipped with a primary UV interference filter. The acidic metabolites were extracted at an acid pH using similar conditions. The urine samples were stabilized by adding 2% sodium metabisulfite to ensure the stability of 4-hydroxypropranolol during collection and storage. Similar reversed-phase methods have been reported for the analysis of propranolol and 4-hydroxypropranolol using fluorescence detection [52,58–59].

VI. VERY-HIGH-SPEED LIQUID CHROMATOGRAPHY

Promising innovations and improvements in column technology have ushered in a new era in therapeutic drug monitoring by liquid chromatography. A series of highly efficient reversed-phase columns (≅100,000 plates/m) packed with 3 or 5 μm packing material, with almost total coverage of the silica base by the alkyl phase, have been introduced recently. The smaller particle packing (3 or 5 μm) results in higher efficiency than conventional packing (10 μm) thus allowing use of shorter columns (3–15 cm long). These shorter columns reduce analysis time because of the smaller column void-volume and the ability to use higher flow-rates (3–5 mL/min) without excessive back pressure. Extracolumn band broadening can be significantly reduced by using shorter and small-bore (0.007-inch) connecting tubing and a detector with a small-volume flow cell (1.4–3.5 μL). These modifications reduce band broadening to about one-fourth of that seen with a conventional liquid chromatograph. Use of a detector with a very fast response time (135 msec) allows for accurate detection of the fast-eluting

analytes [60]. Combined with the use of a modified small-sample loop injection device, these components produce a very efficient and sensitive chromatographic system. Taking advantage of these technological advancements, we have recently developed very-high-speed liquid chromatographic methods for the analysis of theophylline [61], antiepileptic drugs [62], and antiarrhythmic drugs [41].

Two simple isocratic methods, which differ in the composition of their mobile phases and sample preparation techniques, were developed for the analysis of procainamide, NAPA, lidocaine, quinidine, and disopyramide [41]. Procainamide and NAPA can be analysed using a mobile phase consisting of acetonitrile:tetrahydrofuran:20 mmol/L phosphate buffer (9:2:89, v/v/v). The drugs were extracted from 100 µL of serum at alkaline pH into 1.0 mL of methylene chloride. After back-extracting the drugs into 200 µL of 0.1% phosphoric acid, a 20-µL aliquot was injected onto an Ultrasphere C_8 (150 × 4.6 mm) column. The column was eluted with the mobile phase at the flow-rate of 4.0 mL/min, and column effluent was monitored at 280 nm. Under the described conditions, the chromatography was complete in about 1.6 min with good recoveries and precision (Fig. 6). No interference was observed from any of the more than 50 drugs injected into the chromatograph.

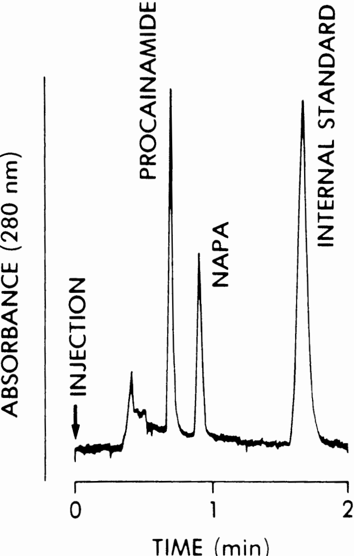

Figure 6 Chromatogram for a patient's serum containing 5.6 mg/L of procainamide and 3.5 mg/L of NAPA under isocratic very-high-speed liquid chromatographic elution.

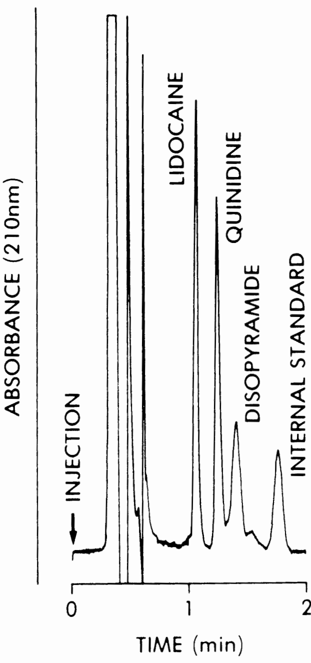

Figure 7 Chromatogram of a standard mixture of drugs run under very-high-speed liquid chromatography (pronethalol as internal standard).

Quinidine, lidocaine, and disopyramide were extracted from serum by using Bond-Elut extraction columns: 200 μL of serum was placed on the top of the column with 200 μL of 10 mmol/L Na_2CO_3. The adsorbed drugs were eluted with 200 μL of methanol containing small amounts of acetic acid and triethylamine. An aliquot of this sample was injected onto a Ultrasphere C_8 (150 × 4.6 mm) column, and eluted with a mobile phase consisting of acetonitrile:tetrahydrofuran:phosphate buffer (27:5:68, v/v/v) at a flow-rate of 4.0 mL/min at 50°C. The column effluent was monitored at 210 nm. The chromatography was complete in <2.0 min with good precision and recoveries (Fig. 7).

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III

LABORATORY MANAGEMENT AND MISCELLANEOUS TOPICS

15

MEDICOLEGAL GUIDELINES FOR THE CLINICAL TOXICOLOGY LABORATORY

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I. INTRODUCTION

The primary goal of the clinical toxicology laboratory is to provide timely analytical and interpretive services to aid the medical community in the diagnosis of drug overdose, the management of drug therapy, and in the evaluation of toxicity from a wide variety of commercial, industrial, and environmental agents. As such, the clinical toxicology laboratory is generally not prepared for, nor interested in, medicolegal investigations.

There are, however, many compelling reasons for the clinical toxicologist to be familiar with medicolegal principles. The analytical skills and experience required to achieve the goals of the clinical toxicology laboratory are frequently in great demand by the persons and agencies performing mediocolegal investigations. For this reason, many clinical laboratories occasionally accept forensic casework.

Even if the laboratory chooses to avoid medicolegal cases, it is not possible for most toxicology laboratories to completely avoid involvement in medicolegal arenas. The clinical toxicology laboratory may perform drug screening or blood alcohol analyses solely for diagnostic purposes, but it is not uncommon for these patients to be involved in some legal actions after recovery. In these situations the practices and procedures of the clinical toxicology laboratory may be examined by the court.

The opinions expressed in this chapter are those of the author and do not necessarily represent those of his employers, the editor, or Marcel Dekker, Inc. This chapter is intended as a supplement to, not a substitute for, competent legal advice.

Occasionally the laboratory may commit an error which makes it liable for malpractice. We live in a society increasingly prone to litigation where malpractice action is becoming more prevalent. As new diagnostic testing claims a larger role in medical practice, the malpractice liability of the clinical laboratory has also increased. The clinical toxicology laboratory is especially vulnerable since many toxicological analyses are performed in emergency situations when there may be limited knowledge of the patient's clinical history and where there may be little time for other ancillary information and evaluation to confirm or refute the laboratory findings. The inappropriate use of potentially dangerous antidotal therapy due to a false-positive acetaminophen report, or conversly, the failure to institute such therapy due to a false-negative acetaminophen report are two examples of situations where a malpractice suit may involve the clinical toxicology laboratory. Similarly, the laboratory may share liability if it incorrectly analyzes a therapeutic drug monitoring sample. This is especially true in situations such as antidepressant therapy where the therapeutic effects of the drugs may not be clinically apparent and the laboratory data assume a more significant role in good patient care.

Clinical toxicology laboratories which make themselves available for medicolegal samples as well as those who attempt to avoid such involvement can benefit from some knowledge for medicolegal principles. This chapter reviews some medicolegal aspects of clinical toxicology.

II. THE MEDICOLEGAL ENVIRONMENT

Whenever a patient is injured, there is the possibility of some form of legal action. If negligence by some member of the health care team is implicated, the legal action most frequently involved is a case of malpactice. In addition to the plaintiff's injury, medical malpractice has three additional elements (Table 1), all of which must be established in court in order to obtain a malpractice decision.

The duty to provide good medical care to a patient may be expressed in a formal contract or may be implied. A hospital which provides an emergency room has an implied duty to provide appropriate medical care to all patients who utilize the facility. Similarly, patients who consult

Table 1 The Elements of Negligence or Malpractice

1. The existence of a duty to the plaintiff
2. Failure to maintain a professional standard of care
3. Damage or injury to the plaintiff
4. A direct relationship between the failure or breach of duty and the plaintiff's injury

a private or hospital-based physician do so with the implied understanding that the physician, as well as the laboratory he or she utilizes, has a duty to provide appropriate care and will accept responsibility for a failure to provide such care.

Each medical profession or specialty, including clinical toxicology, has general standards which its members follow. The standards which may be used in a malpractice action are not drawn from any single source, but are a compilation of generally available guidelines. The standards for a clinical toxicology laboratory are partly set forth in guidelines issued by nationally recognized certifying agencies such as the College of American Pathologists or the Health Care Financing Administration. The standards of care for a clinical toxicology laboratory are also impacted by professional societies such as the American Association for Clinical Chemistry and the National Committee for Clinical Laboratory Standards and many others. The methods employed by a clinical toxicology laboratory are judged by standards which are set by the available technology, published advances in analytical technique, and peer review. For example, publication of this volume increases the availability of well-characterized analytical methods, and therefore is part of the process of setting standards for appropriate methods for the toxicology laboratory.

In addition to national standards, each local area sets standards for other aspects of laboratory operation. The speed of analyzing therapeutic monitoring samples and reporting results to physicians is one standard of care which is usually set by local performance. There are many local differences in the availability of emergency drug overdose testing and in some areas, local standards for the types of analyses which are employed. If fast, accurate therapeutic monitoring or overdose toxicology services are available but not used, the treating physician may be liable if the patient is harmed by this omission. If such testing is ordered but analyzed by methods which are not appropriate, or are analyzed in a fashion inconsistent with local practice, the laboratory may be liable.

In some areas of the clinical laboratory, it is difficult to demonstrate a direct connection between a laboratory error and patient harm. In other sections of the laboratory, such as the blood bank, the relationship between an incorrect determination and patient injury is very plain. The clinical toxicology laboratory is similar to the blood bank in that errors in the toxicology laboratory can often be directly associated with mistreatment of a patient. A falsely low peak gentamicin analysis, for example, might be used by a physician to substantiate an increased gentamicin dosing schedule. This error could directly lead to irreversible renal damage or hearing loss. As the medical profession increases its reliance on laboratory analyses for diagnosis and treatment, the direct cause/effect relationship between laboratory error and patient harm becomes increasingly evident. This therefore exposes the clinical toxicology laboratory to increased liability.

In general, laboratorians are held liable for performing their duties with reasonable care. This responsibility includes maintenance of good laboratory practices, adequate knowledge, training, and skill, as well as the use of good judgement in a laboratory.

As can be seen from this brief discussion, there is more to malpractice than a simple laboratory error. If any of the elements of malpractice cannot be proven in court, the case cannot be won. In cases where mental anguish or suffering is claimed, due, for example, to a delayed test result, injury is very hard to prove, and these cases are less frequently settled in favor of the plaintiff. Where physical injury or death is involved, the element of damage to the plaintiff is easy to demonstrate. The other three elements of malpractice may be more difficult to prove. In many cases the standard of care involves knowledge of subjects outside the general knowledge of the court. Expert testimony is often required to determine what constitutes the standard of care in the case. Expert testimony is also frequently required to determine if the standard has been breached by the defendent. Many experts are unwilling to provide this testimony "against" a member of their own profession which makes the proof of malpractice impossible. This is a misapplication of professional loyalty since each member of a profession should be willing to aid in the examination of acts of possible negligence in order to assist in acquittal of the innocent and reprimand of the truly guilty.

Res ipsa loquitor is a doctrine often used to help prove malpractice. Literally it means, "The thing speaks for itself." It is not a proof of negligence by itself, but is often used to imply that the injury itself shows that someone was negligent. In order to be invoked, several elements must be shown: (a) the patient did not contribute to his injury; (b) the negligent act was in the exclusive control of the defendant; and (c) the injury would not have occurred in the absence of some negligence. In some clinical toxicology cases, *res ipsa loquitor* may not apply since this last element requires a fairly long history of successful treatment with no negligence. Many clinical toxicology procedures are so new to medicolegal practice that it may not be possible to demonstrate this history. If *res ipsa loquitor* does not apply, the acts of the defendant must be shown to be the direct or proximate cause of the injury. Sometimes this is done by showing that if the negligent act had not been committed, injury would not have occurred. For example, if the laboratory had not reported an incorrectly low theophylline value, the physician would not have administered more theophylline, and the patient would have avoided drug toxicity.

There are countless ways for a laboratory to become involved in a malpractice case. The possibility of a malpractice suit involving the laboratory is naturally created whenever the laboratory reports an incorrect test result which eventually causes harm to a patient. If it

can be shown that the laboratory has failed to maintain a standard of care, the chances for a judgment against the laboratory increase dramatically. Thus, personnel practices such as the failure to adequately interview job applicants, verify educational background, conduct appropriate in-service training, counsel or dismiss poor performers, or maintain proper staffing levels may render the laboratory liable if an improperly trained employee commits an error. Improper specimen-handling protocols which contribute to misidentification increase the laboratory's liability. The list of examples could also include failure to maintain any of the hundreds of separate standards involved in running a laboratory.

Defining and proving malpractice are difficult but just as difficult and important is defining who is liable and to what degree. There are several concepts which act to limit the personal liability of laboratory workers and increase the liability of their employers. Under the doctrine of *respondeat superior* (often termed the "master of the ship" doctrine), the employer is legally responsible for the actions of all employees even if they are shown to be negligent. This protection for individual laboratory workers only exists within the confines of their job descriptions.

The actions of laboratory workers in analyzing specimens, and calculating and transcribing results are generally viewed as mechanical or administrative acts and therefore completely controlled by the hospital and covered by the hospital's liability. Interpretation of laboratory values or recommendations for changes in dosing are generally functions of the laboraotry director. These opinions may be viewed as medical services and therefore outside the scope of the hospital's coverage.

As the individual primarily responsible for the direct case of a patient, the treating physician bears the greatest individual liability. The physician's liability can be reduced if it can be shown that his or her actions were influenced by an intervening cause such as an erroneous laboratory report. If, however, the physician did not use good medical judgment or acted on grossly erroneous results, he or she may still have to bear a majority of the liability.

Most suits which result from the negligent act of an employee also name the parent organization as a codefendant or perhaps as the only defendant. This is because the employer has "deep pockets," that is, the financial resources to pay a large settlement. In addition, a jury is more likely to award a large judgement against an impersonal organization rather than a lone laboratory technologist.

For these reasons, although there are many sources of malpractice liability, individual technologists are rarely sued for their actions. It is important for the organizations they work for to be aware of the various sources of liability and mechanisms for decreasing their risk.

III. MINIMIZING LIABILITY

Prevention of errors is clearly the best cure for malpractice liability. It is therefore not suprising that the best way to minimize malpractice liability is by adherence to good laboratory standards, as set forth in guidelines issued by certifying agencies such as the College of American Pathologist, Joint Commission on the Accrediatation of Hospitals, Health Care Financing Administration, and the National Committee for Clinical Laboratory Standards. Even if a laboratory is not licensed by these organizations, their recommendations are a significant factor in establishing the standards by which any laboratory would be judged in a malpractice case.

Laboratory directors are well aware of the necessity for well-written analytical procedures. It is just as important to have standard operating procedures for all aspects of the laboratory. The use of technical, administrative, and clerical operating procedures is an excellent way to insure that all staff members have a clear understanding of the laboratory's operations. This will not only help to prevent errors, but will also assist in reducing the number of people involved in medicolegal processing. Clear operating procedures allow one person to properly conduct functions which may be performed very infrequently and therefore make it possible to avoid adding people to the chain of custody. Standard operating procedures tend to minimize the confusion and panic that may accompany the entry of a medicolegal specimen. Periodic review of operating procedures with all employees will increase the confidence that all procedures are being performed uniformly. This is of extreme importance if it becomes necessary to reconstruct a medicolegal chain of custody for a specimen which was initially delivered as a routine clinical specimen. Operating procedures which require documentation of the personnel involved in specimen receipt, storage, analysis, result review, and reporting will generally enable an acceptable reconstruction of the chain.

A comprehensive job description in a medicolegal situation is an enumeration of the job functions for which the employer accepts liability under the doctrine of *respondeat superior*. Whenever an employee performs additional functions in order to minimize the number of involved personnel, it is vital to limit these functions to those for which clear qualifications exist. A laboratory technologist may receive a specimen for analysis even if this is not his or her normal function; however, technologists should avoid interpretation of pharmacokinetic data or recommendations of dosage changes unless their job descriptions and qualifications specifically permit such interpretive duties. A clear table of organization which delineates lines of authority and individual responsibilities is a logical extension of job descriptions and also serves to limit individual liability.

The laboratory can be asked to demonstrate to the court that the analytical method used is appropriate. This demonstration can include

validation of their method by comparison to a standard method, a review of the data which shows the routine precision, sensitivity, linearity, and specificity of their method, as well as inspection of relevant proficiency testing records. Verification of the qualitative and quantitative composition of all primary standards should be performed by each laboratory. It may not be sufficient to accept a manufacturer's statement of identity without conducting further analyses to substantiate their claim.

The laboratory must be able to prove that on the day in question, the analytical method was properly calibrated and in good control. Verification of the proper functioning of all analytical instruments as well as ancillary equipment should be available. The laboratory worksheet is central to proving the validity of testing and should therefore contain as much information as possible. Control values and the acceptable ranges for all controls should appear on each worksheet. In addition, the date of analysis, as well as the personnel performing the testing, and the supervisory review of the work should be recorded. All specimen identification numbers and other pertinent information such as the time of sampling, condition of the specimen, or the use of a nonstandard specimen type should be noted on the worksheet.

The laboratory is responsible for issuing accurate guidelines for obtaining and preserving specimens. Data which document the stability of properly collected specimens as well as a mechanism to identify improper preservation should be available.

Laboratory results should be viewed as confidential information. The results of testing should be released only to the physician or other responsible agency that submitted the sample. No results should be given in response to a phone call unless the person is known to the laboratory personnel. If results must be given by phone, the laboratory should place the call so that the identity of the recipient can be verified.

In addition to the record keeping requirements of a clinical laboratory, medicolegal specimens require some extra measures. All of the physical characteristics of the sample should be noted. Often the color, consistency, or shape of an illicit medication is as important to the case as the identity of the drug. Whenever possible, the original instrument printouts or chromatograms with specimen identification numbers should be retained since they may include information about the analytical run which would ordinarily not be transcribed to laboratory worksheets. The specimens themselves can be considered evidence, and should therefore be retained in a secure location for as long as possible.

Records of medicolegal analyses should be retained for a minimum of six years. This period will cover the record retention guidelines and statutes of limitation for most state and federal regulations. It is important to consider, however, that the statute of limitations does not start until the discovery of injury and/or the release of medical records to the plaintiff. Since these events may be delayed for several years

after the incident, a medicolegal record retention of at least ten years is prudent.

In addition to maintenance of good laboratory practices, there are several legal principles which may also limit liability. Just as someone may implicate a laboratory error as an intervening cause, the laboratory may implicate faulty reagents as an intervening cause of a laboratory error. To do this, of course, the laboratory would have to demonstrate that they had used the material according to all of the manufacturer's instructions. The laboratory would also have to show that the reagents had not expired or been mishandled upon receipt or during storage, and that all other standards of good laboratory practice had been followed.

If it can be shown that the patient contributed to the injury, the laboratory's liability may be lessened. If the patient proceeds with an action which he or she knows, or should have known, would result in injury, then that patient assumes a portion of the liability. The physician is required to inform patients of significant side effects and dangers of drug therapy as well as precautions to minimize toxicity. If the patient consents to therapy, but ignores the precautions, the physician is not liable.

If it appears that a malpractice claim is imminent, there are several "do's and don'ts" to limit liability (Tables 2 and 3). All information required for treatment of the patient should be immediately communicated. This includes notification of an error, missing sample, or other act which may have a bearing on continued care. If the error was due to improper calibration or some other factor which may alter future analyses, immediate corrective action should be instituted. The corrective action should *not* include immediate dismissal of the involved worker, although counseling or reassignment would be appropriate. Immediately, contact legal counsel or the risk officer to obtain further guidance. Begin an investigation of the incident. Question involved personnel and gather together any relevant documentation.

All of the laboratory records as well as any written reports requested by management can be subpoenaed by the court. If you desire the conclusions of your investigation to be protected from disclosure under the attorney-client privilege, the reports have to be designated as

Table 2 Immediate Actions to Limit Liability

1. Transmit all patient information.
2. Initiate corrective action.
3. Contact legal counsel or risk officer.
4. Investigate the incident.

Table 3 Actions to Avoid if a Suit Seems Likely

1.	Volunteering nonpatient information
2.	Admitting liability
3.	Offering opinions
4.	Revealing the nature or amount of insurance coverage

confidential—for the use of attorney only—and retained separately from the remainder of your records of the case.

All personnel should refrain from volunteering nonpatient information, opinions as to how the error occurred, or who was to blame. It is also unwise to admit liability or attempt to negotiate a settlement prior to contacting counsel. Occasionally, revealing tl e nature or amount of malpractice insurance will only encourage the filing of a malpractice claim.

IV. THE MEDICOLEGAL SAMPLE

Proper sample handling is a vital link in preserving the medicolegal value of the toxicology laboratory's analyses. Knowledge of proper sample handling procedures may also minimize the disruption of normal laboratory routine that medicolegal procedures or testimony can produce. As with any other aspect of laboratory operations, a well-written standard operating procedure for medicolegal specimen handling should have been reviewed by all laboratory personnel and should be readily accessible for reference when the need arises. A major goal of this operating procedure is to insure proper preparation of a chain of custody document. A properly designed chain will not only record all transfers of the sample, but will also serve as a checklist for other aspects of sample preparation (Fig. 1).

The major components of a chain of custody are listed in Table 4. All samples should be described in as much detail as possible. The description of blood samples should include the volume drawn and the anticoagulant or preservative employed. For other samples, the number of items, or volume, color, type of containers, and any other distinguishing characteristics should be recorded. Patient name and all identification numbers for the patient, laboratory request slip, or other codes should be indicated on the chain of custody and on the samples themselves.

Medicolegal samples can come from a variety of sources. Suspect household or medicinal products may be delivered by relatives; illicit drugs may be discovered by emergency room personnel or confiscated

MEDICOLEGAL CHAIN OF CUSTODY

1. DESCRIPTION OF SAMPLES: (include number of samples, physical characteristics and amount of each sample, type of containers and any identifying numbers).

   ____
   ____
   ____
   ____
   ____

2. HOW WERE THE SAMPLES OBTAINED?

   ____
   ____

   Samples obtained by ____________________ ____________
   signature date

3. PREPARATION OF SAMPLES FOR SHIPMENT:
   Description of packing containers:

   ____
   ____

   Seal the package with paper tape and sign the tape so that the package cannot be opened without altering the signature.
   Package prepared and sealed by ____________________ ____________
   signature date

4. TRANSMISSION OF SAMPLES:
   Address to:

   Clearly mark exterior of package as a Medicolegal Sample.

Figure 1 Chain of custody document for medicolegal samples.

Table 4 The Chain of Custody Document

1. A complete description of the sample or samples.
2. A complete record of all identifying numbers.
3. How were the samples obtained?
4. Who obtained the samples and when?
5. Who prepared the samples for delivery to the lab?
6. A record of each transfer of the sample including:

   Specimen received from
   Specimen received by
   Date and time

by police officers. The manner in which samples were obtained should be documented by the person who obtained the samples. When blood samples are collected, the type of disinfectant employed and the site of venipuncture should be indicated as well.

All samples should be sealed with a paper seal coated with a non-peelable adhesive. The seal should be signed or initiated prior to being placed securely over the closure of the container. It is vital that the signed seal be placed on the container in such a way that the container cannot be opened without disturbing the seal. Up to this point, everyone who has had access to the samples should have signed the chain of custody document. Once the samples have been properly sealed, persons who handle the sample need not sign the chain of custody document until the seal is opened for analysis. If the sample is to be analyzed in the same facility it was collected in, the sample and chain of custody should be hand-carried to the laboraotry and delivered to the person who will perform the analysis. If the analysis will be delayed, the sample should be stored in a locked refrigerator and a note made on the chain or custody document. In situations where the laboratory may not be staffed at all times, provision for after-hours delivery to the locked storage area must be made.

If the analysis will be performed by an outside laboratory, the sample and chain of custody should be sealed in a second container. This container should be sealed with a signed paper seal in the same fashion as the specimen containers. The package should be addressed to a specific individual at the receiving laboratory and clearly marked as a medicolegal specimen so that it will not be opened by unauthorized parties. The courts have recognized that when properly sealed and delivered by U.S. mail, the sample can be assumed to be unaltered upon receipt.

If the sample is to be hand-delivered to an outside laboratory, each courier could potentially be considered part of the chain of custody. Therefore, samples should be sealed and addressed as above, but the chain of custody document should be available for each courier to sign.

When the sample is received by the laboratory, the seal should be inspected and its condition noted on the laboratory records. If at any time the sample will be unattended, it should be stored in a limited access area. Whenever a medicolegal specimen is in your custody, it is your responsibility to insure that no one else has access to the sample so that you can prevent the loss or alteration of the sample.

Each chain of custody document must contain a complete record of each person who had access to the specimen (Fig. 2). As each person receives the sample, he or she should sign and date the form and indicate the name and title of the person from whom it was received. There are several sources of collection kits specifically designed to facilitate the proper collection and transportation of medicolegal specimens (Fig. 3). However, it is possible to employ any containers as long as the guidelines presented above are followed.

A. ON__________AT________________I______________________
(date) (time) (signature)

RECEIVED THE SAMPLE DESCRIBED ABOVE FROM:

____
(please print)

B. ON__________AT________________I______________________
(date) (time) (signature)

RECEIVED THE SAMPLE DESCRIBED ABOVE FROM:

____
(please print)

C. ON__________AT________________I______________________
(date) (time) (signature)

RECEIVED THE SAMPLE DESCRIBED ABOVE FROM:

____
(please print)

D. ON__________AT________________I______________________
(date) (time) (signature)

RECEIVED THE SAMPLE DESCRIBED ABOVE FROM:

____
(please print)

Figure 2 Record of access to the medicolegal specimen.

Since anyone with access to the sample may be subpoenaed to testify, the chain should be kept as short as possible. Unless limited by training or job description, each person in the chain should complete as much of the specimen processing as they can. Thus, the phlebotomist who drew a blood sample should fill out the specimen description, label the specimens, and prepare them for transport to the laboratory. These steps should insure efficient and reliable processing of the medicolegal specimen.

V. MEDICOLEGAL TESTIMONY

In conjunction with proper sample handling and appropriate analytical methodology, reliable testimony forms the third leg of a successful medicolegal analysis. Medicolegal testimony can be divided into three

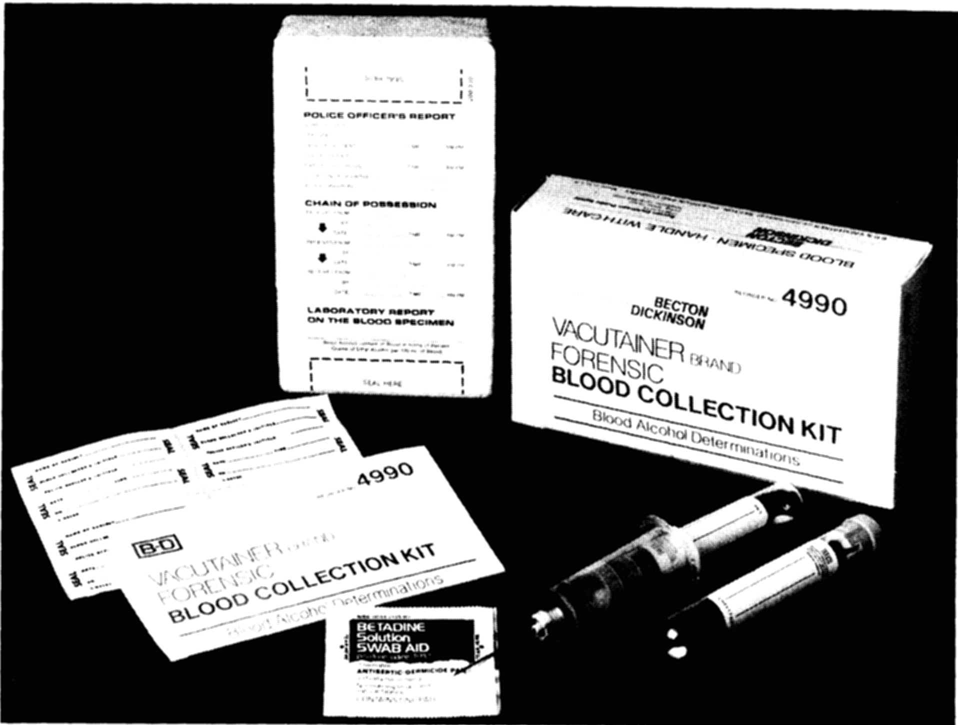

Figure 3 Collection kit for medicolegal specimens.

separate but interrelated categories: evidential, descriptive, and expert.

Evidential testimony is required to personally qualify any evidence including the samples set to the toxicology laboratory. If the chain of custody document and the seals on all samples have been properly maintained, the court may not subpoena all members of the chain. It may only be necessary for the individuals at the beginning and end of the chain to give evidential testimony. Typical questions asked during this type of testimony are: Did you receive a blood sample on that date? Do you remember its condition? Was the sample sealed, and if so, how?

During descriptive testimony, the court will ask for a simple description of what was done and what results were obtained. This testimony can cover not only the analytical procedures, but information regarding administrative and clerical functions as well. It is during this phase of testimony that standard operating procedures are most beneficial. It is occasionally possible for a laboratory director to testify about procedures which he or she did not personally perform,

but which were performed by employees reporting directly to the director, following standard operating procedures that the laboratory director has established and reviewed. Typical questions asked during this type of testimony are: What was done with the specimen after receipt? How are samples logged into the laboratory? What tests were conducted? What were the results of those tests?

Finally, the court may require expert testimony. In most states a physician is the only person the court will qualify as an expert. On occasion, where their training or background gives them special expertise in an area, nonphysician personnel will be allowed to give expert testimony also. Expert witnesses are allowed to interpret results and express their opinions about the facts presented. Frequently, an expert witness was not involved in the case as it occurred, but is presented the facts of the case to review as a consultant for the court. Typical questions asked during expert testimony are: What would that level of alcohol do to a person's driving ability? What blood level would that dose be expected to give?

Courtroom testimony is a unique experience. Although there are many adversary situations in everyday life, there are few outside the courtroom in which you will encounter someone whose job consists of destroying your credibility as a toxicologist! Preparation is your best ally. You should be thoroughly familiar with all records that have been subpoenaed by the court. Your should take to the court only those records you are fairly sure you will need to refer to. Anything you refer to on the stand can be entered into evidence, and therfore it should not contain any extraneous material. Remember that the judge and jury do not generally have extensive technical backgrounds. Give complete answers, but avoid terminology which may be unfamiliar to the general public. Also, avoid expounding on a topic, lest you make an error or confuse the entire courtroom. Avoid volunteering information that is not requested directly.

It is of utmost importance to maintain your objectivity. If you maintain your professional objectivity, your testimony will have far greater impact on the court. The witness who develops a strong opinion about the guilt or innocence of the litigants or who takes personal offense at the style and content of the questions asked is sure to have a difficult and less than effective courtroom experience.

VI. CONCLUSION

There are many ways that a clinical toxicology laboratory may become involved in medicolegal issues. Strict adherence to the standards of good laboratory practice and careful attention to some of the special requirements of medicolegal situations can make the experience a beneficial one for all involved.

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DRUGS AND LABORATORY MANAGEMENT: CLINICAL AND TECHNICAL CONSIDERATIONS

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I. INTRODUCTION

This chapter deals with two major topics: first, the analysis of drugs which have not been reviewed in the previous chapters; and second, various laboratory management considerations.

II. LC DRUG ANALYSIS

For LC drug analyses in TDM and toxicology, the reader may consult the general references: 1–7. Specific references include the following: for abused drugs, i.e., narcotics, hypnotics, and others, Refs. 8–13; for neuroleptics and antihistamines, Ref. 14; for arthritis drugs, Ref. 15; and for steroid, Ref. 16. In order to avoid overlap with these publications, references are limited to post-1980 published LC drug analyses of biological specimens such as serum or urine. Since the major classes of drugs were reviewed in previous chapters, LC analyses of these groups are not included. For clarity, the review will be brief and in table format (Table 1) providing essential information and highlighting the clinical indications, the extraction procedures, column/mobile phase, detection mode, and limit of detection. Readers are strongly encouraged to refer to the original articles for details.

III. LABORATORY MANAGEMENT

The purpose of this section is to consider the personnel, technical, and quality control requirements. Some of these topics were reviewed recently [6,17]. The following discussion is based upon the author's

Table 1 Liquid Chromatographic Measurements of Drugs for TDM and Toxicology[a]

Drug name [Ref.]	Indications	Extraction procedure	Analysis (column/mobile phase)	Detection/limit
1. Acenocoumarin [21]	Anticoagulant	Pet. ether and dichloromethane	Lichrosorb RP 18/ACN + acetate (35:65)	308 nm 15 μg/L
2. Acenocoumarin [22]	Above	Tenax/HOAc	Col-1/ethanol + acetate (1:1)	313 nm 10 μg/L
3. Acetaminophen [23]	Analgesic and antipyretic	Barium hydroxide & zinc sulfate	Col-1/MeOH + water (15:85)	240 nm <1 μg/L
4. Acetaminophen [24]	Above	Ethyl acetate	Col-1/ACN + acetate (3.5:96.5)	254 nm 0.1 mg/L
5. Acetaminophen [25]	Above	Ethyl acetate	Col-1/ACN + phosphate (9.8:90.2)	254 nm 0.5 mg/L
6. Acetaminophen metabolites [26]	Above	β-Glucuronidase	Ultrasphere ODS/MeOH + acetate + phosphate	248 nm 1 ng EC 0.1 ng
7. Acetylsalicylate [25]	Analgesic	—	Refer to 5	Refer to 5
8. Acetylsalicylate [27]	Above	Ether + hexane/phosphate	Spherisorb ODS/water + phosphate + ACN (35:40:25)	234 nm 50 μg/L
9. Allopurinol and a metabolite, oxipurinol [28]	Gout and hyperuricemia	Citrate + phosphate	SAS-Hypersil/citrate + phosphate	252 nm 0.1 mg/L
10. 4-Aminopyridine [29]	Muscle relaxant	Methylene chloride + 1-pentanol	Nucleosil C_{18}/ACN + MeOH + carbonate (61:35:4)	245 nm 1 μg/L

11. Antranfenine and metabol. [30]	Analgesic and antiinflammatory	Ether	Spherisorb ODS/ACN + acetate (72.5:27.5)	353 nm 5 μg/L
12. 1-β-D-Arabinofuranosyl-cytosine (Ara-C) and 1-β-D-arabinofuranosyl-uracil (Ara-U) [31]	Leukemia	Ultrafiltration	Nucleosil C_{18}/phosphate	280 nm 2 μg/L (Ara-C) 264 nm 100 μg/L (Ara-U)
13. Azathioprine [32]	Immunosuppressive	Ethyl acetate	Col-1/phosphate + ACN	280 nm 10 μg/L
14. Benzodiazepines [33]	Sedative	Chloroform + isopropanol	Zorbax ODS/acetate + ACN	254 nm 0.05–0.1 mg/L
15. Benzodiazepines [34]	Above	Chloroform	Col-1/ACN + MeOH + acetate (200:225:500)	240 nm 0.04 mg/L
16. Bisantrene.CL 216,942 [35]	Antineoplastic	Sep-Pak C_{18}/MeOH + water + phosphate/chloroform + MeOH	Col-1/ammonium acetate + MeOH (6:4)	260 nm Ex. 550 nm Em. 2 μg/L
17. Cannabinoids (THC) [36]	(Antiemetic)	MeOH + chloroform	Spherisorb S5 ODS/dil. sulfuric acid/MeOH + ACN (7:8:9)	220 nm —
18. Carbidopa [37]	Parkinson's disease	Alumina	Spherisorb ODS/phosphate + citrate + octanesulfonic acid + EDTA + MeOH	EC 15 μg/L
19. Chlorambucil [38]	Antineoplastic	ACN	Rad Pak C_{18}/ACN + acetate (65:35)	263 nm 1 μg/L
20. Chlordiazepoxide and metabolites [39]	Sedative	Ether	Lichrosorb RP 8/acetate + MeOH + ACN	240 nm 20 μg/L

Table 1 (Continued)

Drug name [Ref.]	Indications	Extraction procedure	Analysis (column/mobile phase)	Detection/limit
21. Chlormethiazole [40]	Sedative	Phosphotungstunic acid	Ultrasphere ODS/ACN + phosphate (45:55)	254 nm 0.25 mg/L
22. Chlorpromazine [41]	Antipsychotic	*n*-heptane + ISAA/dil. HCl + ether/NaOH	Nucleosil C_{18}/pyridine + THF ACN + acetate (0.1:1:68.9:30)	EC 100 pg
23. Cimetidine [42]	H_2-receptor antagonist	Methylene chloride	Rad Pak A/Triethylamine + ACN + water (1:5:94)	228 nm 25 μg/L
24. Cimetidine [43]	Above	ACN	Ultrasphere ODS/phosphate + MeOH (8:2)	220 nm 0.1 mg/L
25. Cimetidine and metabolites [44]	Above	ACN + phosphate/dil. HCl + ISAA/methylene chloride	Zorbax Sil/ACN + MeOH + water + ammonia	228 nm 0.05 mg/L
26. Clobazam [45]	Tranquilizer	Ether	Rad Pak C_{18}/ACN + phosphate (45:55)	254 nm 20 μg/L
27. Clonazepam [46]	Antiepileptic	Chloroform	Spherisorb C_6/ACN + acetate (4:6)	306 nm —
28. Clonazepam [47]	Above	Ether	Col-1/ACN + water (4:6)	254 nm 10 μg/L
29. Cloprednol [48]	Collagen and allergic disease	Methylene chloride + ether (4:6)	Lichrosorb Si 60/methylene chloride + EtOH + HOAc (96:4:0.01)	254 nm —
30. Cocaine and metabolites [49]	Stimulant	Chloroform + isopropanol	Col-1/water + ACN + MeOH (8:1:1) with 1% HOAc and 0.3 M EDTA	235 nm <1 mg/L
31. Cocaine [50]	Stimulant	Ether/HOAc/ *n*-hexane	ODS-HC SIL-X-1 (C_{18})/MeOH + phosphate (75:25)	232 nm --

32. Codeine [51]	Analgesic	Hexane + methylene chloride	Col-1/MeOH + phosphate (21:79)	213 nm EX 4 μg/L
33. Cyclosporin A [52]	Immunosuppresive	Ether/MeOH + *n*-hexane/ether	LC_{18}/ACN + water (68.5:31.5)	202 nm 25 μg/L
34. Cyclosporine [53]	Above	Cyano col./MeOH + water	Ultrasphere/TFA + ACN (gradient)	205 nm 50 μg/L
35. Dantrolene and 5-hydroxydantrolene [54]	Muscle relaxant	Ethyl acetate	Lichrosorb RP 18/MeOH + acetate (1:1)	310 nm 0.03 mg/L
36. 2'-Deoxycytidine 5'-triphosphate and cytosine ARA 5'-triphosphate [55]	Leukemia	Perchloric acid + KOH/periodate oxidation	Partisil SAX10/phosphate + MeOH (gradient)	254 nm and 280 nm —
37. Dexamethasone [56]	Antiinflammatory	Heptane/dichloromethane	Col-1/acetate + MeOH + 1-butanol + water (11:19:30:440)	254 nm 10 μg/L
38. DHAQ, CL232 315 [57]	Antitumor	Chloroform	Col-1/ammonium formate + ACN + water (2:1:1)	546 nm —
39. 2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido-[2,3-d]pyrimidine [58]	Antineoplastic	Sep Pak + methylene chloride + MeOH	Zorbax TMS/1-octanesulfonic acid + ACN (65:35)	254 nm 5 μg/L
40. Diazepam and metabolites [59[	Anxiolytic sedative	Bond-Elut/Water + MeOH	FAST-LC-C_8/MeOH + phosphate + ACN	240 nm 25 μg/L

Table 1 (Continued)

Drug name [Ref.]	Indications	Extraction procedure	Analysis (column/mobile phase)	Detection/limit
41. Diethyldithiocarbamate (disulfiram metabolite) [60]	Alcoholism treatment	Chloroform + ACN	C_8/ACN + acetate buffer (65:35)	276 nm 3 μg/L
42. Difunisal [61]	Analgesic	Acetone	Hypersil ODS/nitrate + isopropanol + ethyl acetate (55:25:20)	251 nm 0.5 mg/L
43. Dipyridamole [62]	Antithrombotic	Ether	Ultrasphere C_{18}/ACN + phosphate with 0.01 TMD	280 nm 2 μg/L
44. Disulfiram and metabolites [63]	Alcoholism	Ethyl iodide/zinc sulfate and ether/ACN	μBondapak phenyl/ACN + water (gradient)	280 nm 25 μg/L
45. Daunorubicin and metabolites [64]	Antitumor	—	Col-1/ACN + acetate	EC 10 μg/L
46. Ergot alkaloids [65]	Migraine, uterus-contracting agent	Cyclohexane-butanol (9:1)	Hypersil ODS/ACN + carbonate	328 nm EX 100 ng/L
47. Etomidate [66]	Hypnotic	Pentane	Ultrasphere ODS/ACN + MeOH + water (35:32,5:32.5)	248 nm 2 μg/L
48. Fenbufen and metabolites [67]	Antiinflammatory	MeOH	MicroPak CN10/water + isopropanol + ACN + phosphate (84.5:10:5:0.5)	265 nm 0.5 mg/L
49. Flufenamic acid and mefenamic acid [68]	Analgesic & antiinflammatory	Carbon tetrachloride	μBondapak CN/water + ACN + HOAc (6:3:1)	254 nm 1 mg/L

50. Fenoprofen [69]	Antiinflammatory	Butyl chloride + dil. NaOH	μBondapak alkylphenyl/ACN + water + HOAc (50:50:2)	272 nm 0.5 mg/L
51. 5-Fluorouracil [70]	Anticancer	Perchloric acid	Col-1/phosphate	254 nm 0.1 μmol/L
52. Flurazepam [71]	Hypnotic	Glusulase/ether	Partisil PXS/methylene chloride + MeOH + water + ammonium hydroxide	254 nm 0.5 mg/L
53. Ftorafur and uracil [72]	Anticancer	Chloroform	Zorbax SIL/ethylene chloride + ethanol (24:1)	254 nm 1 μg/L
54. Glafenine, glafenic and hydroxyglafenic acid [73]	Analgesic	MeOH + ammonia solution	Spherisorb R-Sil C_{18}/MeOH + water + acetic acid (67:23:10)	360 nm 0.2 mg/L
55. Haloperidol and metabolite [74]	Neuroleptic	Hexane + ISAA/HCl/NaOH/Hexane + ISAA	μBondapak CN/ACN + phosphate (45:55)	EC 0.25 μg/L
56. Haloperidol [75]	Above	Hexane + ISAA/dil. HCl	Bio-sil ODS-10/ACN + phosphate (4:6)	195 nm p μg/L
57. Haloperidol [76]	Above	Ether + dil. HCl/NaOH + chloroform	Nucleosil C_{18}/MeOH + acetate (63:37)	250 nm 2 μg/L
58. Heroin (3,6-diacetylmorphine) [77]	Narcotic agonist	Toluene-butanol/dil. sulfuric acid + hexane/toluene + butanol	Micropak Si/ACN + MeOH + ammonia + acetate	218 nm 12.5 μg/L
59. 9-(2-hydroxyethoxymethyl)guanine (Acyclovir) [78]	Antibiotics-Herpes	Ammonium sulfate	Zorbax ODS/acetate + heptanesulfonic acid	254 nm 10 μm

Table 1 (Continued)

Drug name [Ref.]	Indications	Extraction procedure	Analysis (column/mobile phase)	Detection/limit
60. Ibuprofen [79]	Antiinflammatory antipyretic, and analgesic	Methanol	Col-1/ACN + HOAc (55:45)	254 nm 0.1 mg/L
61. Ibuprofen [80]	Above	Hexane	ODS-Dupont/MeOH + acetate (65:35)	253 nm Ex. 230–420 nm Em., 1 mg/L
62. Ibuprofen and metabolites [81]	Above	Methylene chloride	Partisil 10 ODS-3/ACN + water or phosphate	220 m 5 mg/L
63. Indomethacin [82]	Prostaglandin inhibitor	Ether	Rad Pak C_{18}/ACN + acetate (45:55)	260 nm 0.1 mg/L
64. Indomethacin [83]	Above	Heptane + ISAA	Col-1/MeOH + water (66:34)	254 nm 0.25 mg/L
65. Isoniazid acetylisoniazid [84]	Above	*n*-butanol + chloroform/dil. H_2SO_4	Col-1(5 μm)/Dioctyl sulfosuccinate in water + ethanol (55:45)	254 nm 0.1 mg/L
66. Laxative by TLC (refer to the original article) [85]				
67. Levodopa [37]	Parkinson's disease	Refer to 18	Refer to 18	Refer to 18
68. Melphalan [86]	Antitumor	Methanol/Sep Pak C_{18}	Spherisorb ODS/MeOH + water (4:6)	260 nm Ex. 350 nm Ex. 500 pg

69. Mercaptopurine [32]	Antineoplastic agent	Refer to 13	Refer to 13	Refer to 13
70. Mercaptopurine [87]	Above	TCA	Spherisorb ODS/phosphate	280 nm and 312 nm 3 μg/L
71. Metformin [88]	Diabetes mellitus	TCA	Partisil SCX/phosphate	230 nm 0.1 mg/L
72. Methimazole [89]	Hyperthyroidism treatment	Chloroform	μBondapak-NH_2/chloroform	405 nm 5 μg/L
73. Methotrexate and 7-hydroxymethotrexate [90]	Anticancer	C_{18} Sep Pak/MeOH + buffer	Col-1/EDTA + acetate + MeOH + ACN	313 nm —
74. Above [91]	Above	Sep Pak C_{18}/MeOH + acetate	Rad Pak C_{18}/ACN + phosphate (15:85)	305 nm 0.06 μM to 0.12 μM
75. Methoxsalen [92]	Psoriasis	Methylene chloride	Lichrosorb 10 RP 9/MeOH + ACN + water (2:30:68)	254 nm 10 μg/L
76. Methyl CCNU [93]	Anticancer	Pentane	Spherisorb ODS/ACN + acetate (1:1)	230 nm 0.2 mg/L
77. 5-Methyltetrahydrofolate [94]	Anticancer	Sodium carbonate	Lichrosorb RP 18/tetrabutylammonium phosphate + MeOH	295 nm Ex. 350 nm Em.
78. Misonidazole and metabolite [95]	Radiosensitizing agent	MeOH + ACN	Ultrasphere/phosphate + ACN (93:7)	313 or 323 nm 0.2 mg/L
79. Morphine [96]	Analgesic and muscle relaxant	Chloroform	Col-1/MeOH + heptanesulfonic acid	EC 1 μg/L

Table 1 (Continued)

Drug name [Ref.]	Indications	Extraction procedure	Analysis (column/mobile phase)	Detection/limit
80. Nalbuphine [97]	Analgesic	Perchloric acid/ethyl acetate + 2-propanol	Biophase ODS/phosphate + MeOH (55:45)	EC 1 ng
81. Naproxen and desmethylnaproxen [98]	Antiinflammatory	Methylene chloride	Zorbax ODS/MeOH + acetate (55:45)	253 nm —
82. Nicotinic acid, nicotinuric acid, and nicotinamide [99]	Hyperlipemia treatment	Acetone/chloroform	Zipax SCX/phosphate	260 nm 0.05 mg/L
83. Nitrazepam [100]	Hypnotic	Ether	Spherisorb S5W/MeOH + ether + perchloric acid (80:20:0.02)	280 nm 5 μg/L
84. Pemoline [101]	Hyperactive children	Methylene chloride	Col-1/ACN + phosphate (17:83)	215 nm 0.5 mg/L
85. Pentamethylmelamine [102]	Anticancer	Ethyl acetate	Col-1/ACN + phosphate (gradient)	240 nm 300 μg/L
86. Penicillamine [103]	Cystinuria and arthritis	TCA	Zipax SCX/citrate + phosphate	EC —
87. D-Penicillamine [104]	Above	Fluorescence derivat.	Lichrosorb RP 18/ACN + phosphate (1:2) with ethylenediamine	338 nm Ex. 430 nm Em. 50 ng
88. Phenprocoumon [105]	Anticoagulant	Dichloroethane	Lichrosorb RP 18/ACN + water + acetic acid (4:6:0.05)	313 nm 0.1 mg/L
89. Phenylpropanolamine [106]	Sympathomimetic agent	Butyl chloride + 1-butanol	ODS(LDC)/ACN + acetate (4:6)	230 nm Ex. 280 nm Em. 1 μg/L

90. Pilocarpine [107]	Topical ocular agent	Methylene chloride/ *p*-nitrobenzylbromide + ACN	Col-1/MeOH + octanesulfonate (8:2)	254 nm <0.05 mg/L
91. Piracetam [108]	Blood disorder	Methanol/Centriflo CF filter	Col-1/phosphate	208 nm 0.2 mmol/L
92. Piroxicam [109]	Antiinflammatory agent	Chloroform	RP 8/phosphate + ACN (6:4)	330 nm 0.5 mg/L
93. Proglumide [110]	Anticholinergic agent	Chloroform	Zorbax SIL/chloroform + MeOH (24:1)	240 nm 0.05 mg/L
94. Promethazine [111]	Neuroleptic	*n*-heptane + dichloromethane + ISAA/dil. HCl	Hypersil 5-SAS/MeOH + Sorensen's buffer	248 nm 2 ng
95. Promethazine [112]	Above	Hexane + ethyl acetate/ EtOAc + trichloroethyl chloroformate	MCH-10/MeOH + water (84:16)	254 nm 1 μg/L
96. Pyridostigmine [113]	Myasthenia gravis	Sep Pak/water + MeOH	Col-1/octanesulfonic acid + MeOH	270 nm 40 μg/L
97. Ranitidine [114]	H_2-receptor antagonist	Methylene chloride	Col-1/MeOH + phosphate (75:25)	330 nm 5 μg/L
98. Salbutamol [115]	Bronchodilator	Sep Pak/MeOH + water	Partisil SCX/phosphate Lichrosorb RP 2/perchlorate + 2-propanol + phosphate	EC 0.5 μg/L
99. Strychnine [116]	Nonketotic hyperglycinemic infants	Chloroform	Silica gel/ammonium hydroxide + MeOH (0.75:99.25)	254 nm 20 mg/L
100. Tetrabenazine [117]	Huntington's disease	Ether/dil. sulfuric acid/mercuric acetate	Col-1/ACN + acetate (1:1)	265 nm Ex. 418 nm Em. 0.1 μg/L

Table 1 (Continued)

Drug name [Ref.]	Indications	Extraction procedure	Analysis (column/mobile phase)	Detection/limit
101. Tetrahydroamino-acridine [118]	Anticholinergic agent	Chloroform	Partisil (Whatman)/TEAP + ACN (75:25)	240 nm 10 μg/L
102. 6-Thioguanine [119]	Antineoplastic agent	Ultrafiltration	Nucleosil/citrate + phosphate	343 nm 0.2 mg/L
103. Thiopental [120]	Intracranial hypertension and anesthetic	*n*-butyl chloride/ NaOH	C_{18}/phosphate + THF (86:14)	240 nm 1 mg/L
104. Thiopental [121]	Above	Chloroform	Col-1/phosphate + ACN + THF (78:22:4)	254 nm 0.1 mg/L
105. Thiopentone [122]	Above	Ethyl acetate	Lichrosorb C_{18}/MeOH + water (6:4)	290 nm 0.2 mg/L
106. Thioridazine and metabolites [123]	Neuroleptic	Ether + hexane/dil. HCl/ether + hexane	Rad Pak Sil/2,2,4-trimethylpentane + methylene chloride + MeOH with methylamine (8:1:1)	254 nm 10 μg/L
107. Thiothixene [124]	Neuroleptic	Sep Pak/hexane + isopropanol (4:1)	Rad Pak CN/MeOH + ACN + phosphate + triethylamine (400:50:50:1)	254 nm 5 μg/L

108. Tiaprofenic acid [125]	Antiinflammatory agent	TCA/chloroform	Hypersil ODS/MeOH + acetic (1:1)	313 nm 0.5 mg/L
109. Tolbutamide and carboxytobutamide [126]	Hypoglycemic agent	Ether	Col-1/ACN + phosphate (35:65)	254 nm 2 or 0.1 mg/L
110. Tolmetin [127]	Antiinflammatory agent	Methylene chloride	Zorbax ODS/MeOH + acetate (6:4)	254 nm 1 mg/L
111. Trimeprazine [128]	Neuroleptic	Pentane + isopropanol	Zorbax CN/ACN + acetate (9:1)	EC 0.13 μg/L
112. D-Tubocurarine [129]	Anesthetic	Ethylene dichloride/dil. HCl	Rad Pak C_{18}/MeOH + triethylamine + Pic B5 + phosphoric acid + water	280 nm 0.02 mg/L
113. Verapamil [130]	Antianginal antiarrhythmic	MeOH	Hypersil C_8/MeOH + acetate (55:45)	278 nm Ex. 320 nm Ex. 5 μg/L
114. Warfarin [131]	Prothombin	Ether/NaOH/dil. sulfuric acid/ether	Col-1/MeOH + acetate (7:3)	308 nm 0.06 mg/L
115. Zomepirac [132]	Antiinflammatory agent	Ether	Col-1/MeOH + acetate (7:3)	330 nm 0.05 mg/L

[a]Nonstandard abbreviations: ACN, acetonitrile; Col-1, μBondapak C_{18}; EC, electrochemical detection; Ex. and Em., excitation and emission wavelength in nm; ISAA, isoamyl alcohol; MeOH, methanol; TCA, trichloroacetic acid; THF, tetrahydrofuran; TFA, trifluoroacetic acid; and HOAc, acetic acid.

experience, with the assumption that a dedicated group of technologists is involved in TDM and toxicology measurements, as explained in Chap. 1. Appropriate modifications of these considerations should be adapted to a particular laboratory.

A. Personnel

Due to the STAT demand of toxicology and some TDM measurements, it is inherently important that the laboratory staff be responsive, providing a fast turnaround-time (TAT) as well as extensive time coverage. Depending on the request, the TAT for lidocaine should be 0.5 hr; for acetaminophen, 1 hr; and several hours for other drugs such as tricyclic antidepressants, and for solvent screening for methanol and ethylene glycol. With the presently available instrumentations described in Chap. 3, these proposed TATs are realistic and technically achievable. Extensive coverage should be provided by two or more working shifts and partial weekend coverage. For example, on weekdays, the author's laboratory is staffed between 8:30 AM and 1:00 AM on the following morning. The hours of 1:00 AM to 8:30 AM are covered by an on-call arrangement. During Saturday and Sundays, the laboratory is staffed with part-time help or on-call coverage. Thus the total time coverage with technologists is estimated to be around 60–70%. As discussed in Chap. 1, personnel should be periodically updated with various aspects of clinical pharmacology and instrumentation for an efficient operation.

B. Technical

The technical considerations may be subdivided into instrumentation, sample preparation, and quality control/surveys. Instrumentation used in TDM/toxicology may range from simple flame photometers to complicated LC and GC. In ensuring adequate operational standards, a rigid preventive maintenance (PM) should be implemented to avoid downtime. (If possible, a backup instrument should be available.) This practice of preventive maintenance is required for the accreditation by the College of American Pathologists. The maintenance protocol may be developed according to the instrument manufacturer's guidelines and the laboratory's experience. The program should be reviewed annually for efficacy and possible avoidance of redundance. A sample sheet for periodic maintenance of LC is provided in Fig. 1. Depending on the laboratory's work load, the procedure should be redesigned accordingly. With such a program, minimum downtime may be achieved. From the author's experience, LC instrumentation will always present minor, unavoidable problems such as leaks, plugged columns, deterioration of pump seals, etc. By training the technologists to watch for these problems, major interruptions may be avoided. For this purpose, it would be worthwhile to train one or two technologists

DRUG ANALYSIS DIVISION - PREVENTIVE MAINTENANCE PROGRAM

CHROMATOGRAPHIC INSTRUMENTS

INSTRUMENT: ______________ MONTH: ______________

ID#	1st Week	2nd Week	3rd Week	4th Week	5th Week
COLUMN CHANGE (4 to 12 months)					
DETECTOR CHECK (Biweekly)					
GUARD COLUMN CHANGE (Biweekly)					
INJECTOR SEAL CHANGE					
K' CHECK & PEAK HEIGHT (Biweekly)					
LUBRICATION					
PUMP SEAL CHANGE					
FLOW RATE & PRESSURE DROP (Weekly)					
LEAK CHECK (Daily)					
SYRINGE CLEANING (Daily)					
SYSTEM AND AREA CLEAN (Daily)					
TEMPERATURE CHECK (Biweekly)					
OVERALL SYSTEM MAINTENANCE					
OTHERS					

Figure 1 Liquid chromatography preventive maintenance program.

to be familiar with a certain instrument. By systematically sharing these duties, the PM program might be readily reinforced by the personnel.

Due to possible interference by other drugs or endogenous compounds, it would be helpful to have available a backup instrumentation, such as FPIA for theophylline, supplemented by an HPLC procedure if necessary.

The extent of sample preparation depends on the method: simple or minimum for immunoassays, to 3-4 steps for chromatographic assays. The following discussion focuses on the more demanding preparations for chromatographic analysis. As discussed in the chapter dealing with the analysis of major classes of drugs (Chap. 3), a typical LC assay would include the preparation of a calibration standard and the addition of internal standard to enhance precision. The number of calibration standards required would depend on the assay. For a simple assay such as theophylline with one-step extraction, one or two calibration standards would suffice; whereas for the more difficult assay, such as the antidepressant (especially if performed by an inexperienced technologist), several standards should be used to generate a calibration curve. As that technologist becomes experienced, the number of standards might be reduced to enhance efficiency without sacrificing precision. The matrix and the stability of the standard should be carefully evaluated by a long-term precision study. For example, the author's experience showed that trazodone standard should be prepared in plasma to afford maximum stability. The use of an internal standard is a good laboratory practice and has been recently reviewed and cautioned by Curry and Whelpton [18]. Generally, the internal standard should have a structure similar to that of the drug, assuring similar, but not identical, extraction efficiency and chromatographic behavior. The author feels that it is more important that the drug and the internal standard exhibit identical chemistry. The problems associated with the use of internal standard are outweighed by the ease and precision evidenced in modern LC drug assays, as shown in the plot for amoxapine and its hydroxylated metabolites (Fig. 2). After the analyses, the peak height (drug/internal standard) ratios are then plotted against the drug concentrations. The patient's drug concentrations may be estimated from this plot.

If a suitable internal standard cannot be obtained, then an external standard approach is used. Caution must be taken to assure quantitative extraction, transfer, and injection, in order that the peak height or the peak areas may be used for constructing the calibration curves. In short, this approach is the least desirable due to possible fluctuation in extraction and chromatography. Alternatively, a radioactive internal standard might be used, but this practice, because of waste disposal problems and time-consuming counting, is seldom used in clinical laboratories today.

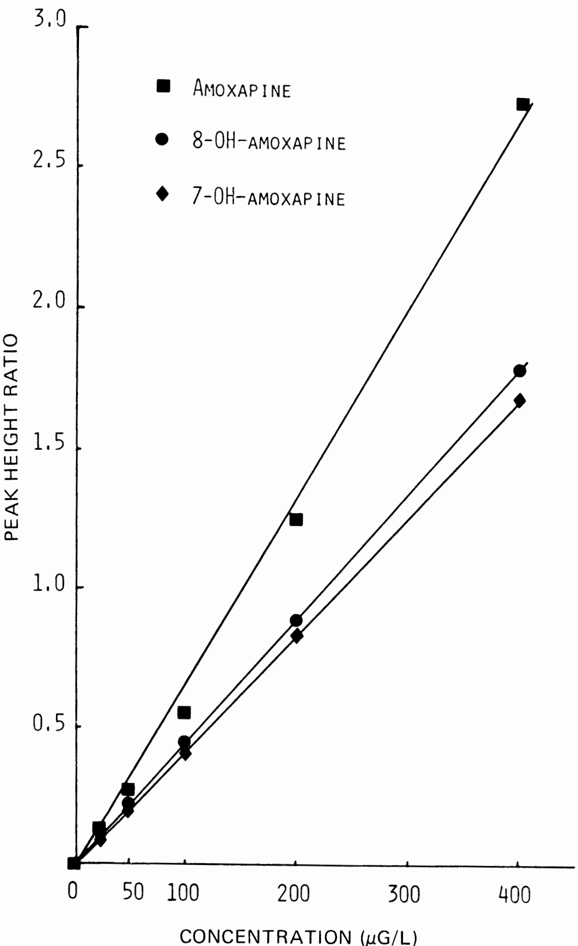

Figure 2 Amoxapine and its hydroxylated metabolites.

Another technical consideration is the kind of sample. Other pertinent information includes ingestion time and condition of the patient. This information is important for the clinical interpretation of laboratory results by the laboratory director, as well as for possible pharmacokinetic interpretation/calculation later on. The information should be entered on the requisition form and on laboratory reports exemplified by the forms shown in Figs. 3a–d. The common specimens are serum, plasma, and urine, followed by cerebral spinal fluid (CSF), saliva, and vomitus. The majority of TDM/toxicology measurements are performed with the first two types. The sampling problems for blood

UNIVERSITY OF CONNECTICUT HEALTH CENTER

DRUG ANALYSIS

Requisition

DEPARTMENT OF LABORATORY MEDICINE

Patient Identification

Physician:	Diagnoses:	STAT	Location:	Obtained by:	Date:	Time: a.m. p.m.

TOXICOLOGY SCREENING TESTS (QUALITATIVE)

SPECIMEN: ☐ BLOOD ☐ URINE ☐ OTHER(specify)__________

(Please fill out as completely as possible)

PROBABLE DATE AND TIME OF INGESTION:__________

SUSPECTED DRUGS OR POISONS:__________

CURRENT DRUG USE OR THERAPY:__________

OTHER PERTINENT INFORMATION:__________

STATE OF CONSCIOUSNESS: AWAKE ☐ DELIRIOUS ☐ LETHARGIC ☐ COMATOSE ☐ INTOXICATED ☐ HALLUCINATING ☐

"TOXI A" SCREENING TEST: ☐ D040

ACETAMINOPHEN
AMITRIPTYLINE
AMPHETAMINE
CAFFEINE
CHLORPROMAZINE
COCAINE
CODEINE
DIAZEPAM
DOXEPIN
IMIPRAMINE
MEPERIDINE
MEPROBAMATE
METHADONE
METHAMPHETAMINE
METHAQUALONE
MORPHINE
NICOTINE
NORTRIPTYLINE
PHENCYCLIDINE
PROPOXYPHENE
QUININE/QUINIDINE
STRYCHNINE
TRIFLUOPERAZINE
TRIFLUOPROMAZINE
TRIMERPRAZINE
OTHER:__________

"TOXI B" SCREENING TEST: ☐ D045

AMOBARBITAL
APROBARBITAL
BARBITAL
BUTABARBITAL
DIAZEPAM
ETHINAMATE
GLUTETHIMIDE
PENTOBARBITAL
PHENOBARBITAL
PHENYTOIN
SECOBARBITAL
OTHER:__________

QUANTITATIVE ASSAY OF DRUGS AND POISONS

Drug	Code	Drug	Code	Drug	Code
ACETAMINOPHEN	☐ D010	ETHOSUXIMIDE	☐ D050	**PHENYTOIN**	☐ D105
ACETONE	☐ D005	ETHYLENE GLYCOL	☐ D056	PRIMIDONE	☐ D110
ALCOHOL	☐ D015	HALOPERIDOL	☐ D061	PROCAINAMIDE/NAPA	☐ D115
AMITRIPTYLINE/NORTRIPTYLINE*	☐ D018	IMIPRAMINE/DESIPRAMINE*	☐ D062	PROPRANOLOL*	☐ D117
AMOXAPINE/8-OH AMOXAPINE*	☐ D021	ISOPROPANOL	☐ D065	QUINIDINE	☐ D120
CANNABINOIDS (URINE REQUIRED)	☐ D026	LIDOCAINE	☐ D070	**SALICYLATE**	☐ D125
CARBAMAZEPINE	☐ D030	LITHIUM	☐ D075	**THEOPHYLLINE**	☐ D135
CHLORAMPHENICOL	☐ D032	MAPROTILINE/ DESMETHYLMAPROTILINE*	☐ D078	TRAZODONE*	☐ D141
DESIPRAMINE	☐ D230			TRIMIPRAMINE*	☐ D142
DIAZEPAM/CHLORDIAZEPOXIDE	☐ D033	METHANOL	☐ D090	VALPROIC ACID	☐ D145
DISOPYRAMIDE	☐ D035	NORTRIPTYLINE*	☐ D091	OTHER:__________	
DOXEPIN/DESMETHYLDOXEPIN*	☐ D038	**PHENOBARBITAL**	☐ D095	__________	

*SPECIAL COLLECTION REQUIRED CALL EXT. 3131

(a)

Figure 3 Requisition form (a) and three reports: one for drug screening (b) and two for drug concentration (c,d).

DRUG SCREEN REPORT
DRUG ANALYSIS DIV.

HCH-458 NEW 9/82 State of Connecticut License No. PH-0213

UNIVERSITY OF CONNECTICUT HEALTH CENTER
Department of Laboratory Medicine
Farmington, CT. 06032

☐ **STAT** ☐ **ROUTINE**

TIME/DATE DRAWN	DATE REPORTED	UNIT NUMBER	ADMISSION NO.	PHYSICIAN	LOCATION

TOXI – A SCREEN (Analgesic/Stimulant/Tranquilizer)

☐ Serum ☐ Urine ☐ Other ________ ☐ **NEGATIVE** Drug(s) listed below were not detected ☐ **POSITIVE** Drug(s) checked below were detected

☐ Acetaminophen	☐ Codeine	☐ Methadone	☐ Propoxyphene
☐ Amitriptyline	☐ Diazepam	☐ Methamphetamine	☐ Quinine +/or Quinidine
☐ Amphetamine	☐ Doxepin	☐ Methaqualone	☐ Strychnine
☐ Caffeine	☐ Imipramine	☐ Morphine	☐ Trifluoperazine
☐ Chlorpromazine	☐ Meperidine	☐ Nicotine	☐ Trifluopromazine
☐ Cocaine	☐ Meprobamate	☐ Nortriptyline	☐ Trimeprazine
		☐ Phencyclidine	

☐ Presumptive Evidence of:

TOXI – B SCREEN (Barbiturate/Sedative)

☐ Serum ☐ Urine ☐ Other ________ ☐ **NEGATIVE** Drug(s) listed below were not detected ☐ **POSITIVE** Drug(s) checked below were detected

☐ Amobarbital	☐ Butabarbital	☐ Glutethimide	☐ Phenytoin
☐ Aprobarbital	☐ Diazepam	☐ Pentobarbital	☐ Secobarbital
☐ Barbital	☐ Ethinamate	☐ Phenobarbital	

☐ Presumptive Evidence of:

SERUM DRUG CONCENTRATIONS:

ETHANOL –

SALICYLATE –

COMMENTS:

TIME OF ORAL REPORT A.M. P.M.	REPORTED TO	REPORTED BY

Steven H. Wong, Ph.D., LABORATORY DIRECTOR

(b)

Figure 3 (Continued)

DRUG CONCENTRATION IN SERUM REPORT

DRUG ANALYSIS DIVISION

HCH-457 Rev. 8/83 State of Connecticut License No. PH-0213

UNIVERSITY OF CONNECTICUT HEALTH CENTER

Department of Laboratory Medicine

	LOCATION										
	PHYSICIAN										
DATE	Drawn										
	Received										
TIME	Drawn										
	Received										
ANALYSIS	Therapeutic Range	Access	No.								
Ethanol mg/dl	Intox = 100										
Salicylate mg/dl	3—30										
Phenytoin mg/L	10—20										
Phenobarbital mg/L	15—40										
Lithium mEq/L	0.7—1.5										
Carbamazepine mg/L	8—12										
Ethosuximide mg/L	40—100										
Primidone mg/L	5—12										
Quinidine mg/L	2—6										
Valproic Acid mg/L	50—100										
Acetaminophen mg/L	Not Applicable										
Theophylline mg/L	8—20										
N-Acetyl Procainamide mg/L	5—30										
Procainamide mg/L											
Chloramphenicol mg/L	10—20										
Cannabinoid (urine)	presumptively positive/negative										

Comments

* Call Lab for Therapeutic Range

Steven H. Wong, Ph.D. Director

(c)

Figure 3 (Continued)

DRUG CONCENTRATION IN SERUM REPORT

DRUG ANALYSIS DIVISION

HCH-457 Rev. 8/83 State of Connecticut License No. PH-0213

UNIVERSITY OF CONNECTICUT HEALTH CENTER

Department of Laboratory Medicine

	LOCATION										
	PHYSICIAN										
DATE	Drawn										
	Received										
TIME	Drawn										
	Received										
ANALYSIS	Therapeutic Range	Access	No.								
Desipramine µg/L	50—250										
Nortriptyline µg/L	50—150										
Imipramine µg/L	150—250										
Desipramine µg/L											
Amitriptyline µg/L	120—250										
Nortriptyline µg/L											
Doxepin µg/L	75—200										
Desmethyldox. µg/L											
Maprotiline µg/L	100—400										
Desmethylmap. µg/L											
Amoxapine µg/L	> 100										
8—OH-Amoxapine µg/L											
Trazodone µg/L											
Trimipramine µg/L											
Atenolol µg/L											
Propranolol µg/L											
4-OH Propranolol µg/L											
Disopyramide mg/L	3.0—7.0										
Lidocaine mg/L	1.0—5.0										
Chlordiazepoxide mg/L	0.4—1.0										
N-Desmethyldiazepam mg/L	0.2—0.5										
N-Deschlordiazeproxide mg/L											
Demoxepam mg/L											
Diazepam mg/L	0.2—0.4										

Comments

* Call Lab for Therapeutic Range

Steven H. Wong, Ph.D. Director

(d)

Figure 3 (Continued)

were reviewed by Lin and Narayanan in Chap. 4, and also by Slockbower [19]. Depending upon the instrumentation/procedures, various types would be needed: for example, plasma is preferred for antidepressant assay due to a serum interference peak, evident from the author's antidepressant procedure. However, serum might be entirely suitable for another laboratory's procedure. Recently, free drug levels have been increasingly utilized for selected patient groups. For the preparation of ultrafiltrate using an Aminco filter, close attention to the timing (within 24 hr) and preparation (temperature and centrifugation requirements) should be followed. Also, CSF and saliva may be suitable for free drug measurements.

C. Quality Control and Surveys

In ascertaining the precision of the measurements, "quality control" (QC) samples are included in clinical drug assays. They should be purchased from established suppliers, if possible, such as Ortho Diagnostics, Fisher, Utak, and others. The merits of quality controls were recently reviewed [6,18,20]. An established policy should be stringently enforced, with periodic review by the director. Interlaboratory quality control assurance may be achieved by performing survey procedures administered by:

1. AACC-TDM
   1725 K Street, N.W.
   Washington, D.C. 20006
2. College of American Pathologists, TDM or Toxicology
   7400 Skokie Blvd.
   Skokie, IL 60077
3. Center for Disease Control (CDC)
   Atlanta, GA 30333

For new drugs which are not available from the QC suppliers (such as trazodone), particular attention should be given to the preparation of in-house "QC." Duplicate or triplicate samples should be included.

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17

CONCLUSION: CURRENT STATUS AND FUTURE DEVELOPMENTS

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TDM/toxicology has been firmly established as a subspecialty within the clinical laboratory. The fundamentals, the current instrumentations, the major classes of drugs and their clinical pharmacology, and LC assays have been reviewed. Potentially useful instrumentations and possible new areas of TDM have also been outlined. Since the practice of routine TDM in the clinical laboratory in the seventies, both clinical pharmacological research and instrumentations such as HPLC, GC, EMIT, and FPIA have advanced this subspecialty to another threshold. With the advent of Diagnostic Related Group reimbursement policy, this subspecialty, like other medical fields, requires self-reexamination in order to design a strategy that is compatible with the health-care system. In short, having examined the various instrumentations and classes of drugs which merit measurement, the laboratory medical community is indeed in a position to choose among various methods, seeking a combination that best fits a laboratory's requirements. LC, with its selectivity and low cost, should be reevaluated as a viable, cost-effective drug assay approach.

The following discussion highlights the editor's observations on this volume. In Chap. 2, Pippenger establishes the principles of TDM. In Chap. 3, the editor compares LC to other methods. LC is cost-effective but more demanding, whereas immunoassays are fast, expensive, and may suffer some interference, as seen in the elevated EMIT measurements of phenytoin levels of renal patients. Digoxin interference was identified in some immunoassay measurements of neonatal patients by Hicks et al., and Schneider et al. recently reported intravenous fluorescein interference with FPIA drug measurement. Some of these problems may be solved by using monoclonal antibody for more selective

binding. In Chap. 4, Narayanan et al. had systematically evaluated the efficacy of blood collection devices for drug monitoring. It would be most advantageous if a universal drug collection device were identified or manufactured. The chapter on fluorescence detection (Chap. 7) and related topics has focussed on present as well as potential applications. This technique offers unique selectivity and sensitivity and should be of interest to the laboraotry interested in research, as well as the laboratory geared to routine TDM/toxicology measurements. Computer interfacing, described by Scott in Chap 5, remains an important goal for the laboratory in the 1980s. As noted by Scott, it may be more desirable to achieve a good separation than to resolve peaks using computer programming. LC-MS, discussed in Chap. 6, represents a challenging and vital tool.

In reviewing LC monitoring of major classes of drugs (Part II), anticonvulsants, antiarrhythmics, and antiasthmatics may be cost-effectively measured as a result of the availability of multidrug analyses. Antidepressants and antihypertensives are exclusively quantitated by chromatography, but the clinical efficacy of the plasma concentration awaits further studies. Some antibiotics such as lactams, cephalosporins, and chloramphenicol, may also be adequately quantitated by LC, as pointed out by Miner in Chap. 10. A succinct account of the injected sample-solvent problems has also been included in this chapter.

At present, a new instrumentation, called STRATUS, has been recently introduced by American Dade. The principle is based upon fluorometric enzyme immunoassay. Utilizing TDx, alcohol and glucose may be quantitated by Radiactive Energy Attenuation (REA) by Abbott Labs, based on enzymatic reactions and the subsequent chromogen attenuation of fluorescence. A dry-phase homogenous immunoassay, an ARIS assay (Apoenzyme Reactivation Immunoassay System) using the Seralyzer by Ames Labs, quantitates a drug, such as theophylline, by a competitive binding assay, based on the theophylline FAD conjugate, not bound to the monoclonal theophylline antibody, reactivating apoglucose oxidase. The ease of this technique would enhance home/office drug monitoring. Another newly introduced commercial assay is the neuroleptic radioreceptor assay. The clinical efficacy of these two new assays awaits further study. A dedicated LC analyzer, QA1, as described in Chap. 3, may offer automated LC analysis with minimal personnel involvement. Sample preparation cartridges and apparatus would increase efficiency and enhance extraction recovery.

Looking into the future, TDM/toxicology may expand into the health care of selected patient groups such as oncologic, pediatric, renal, obstetric, and geriatric specialties. Each of these groups place special demands on the capacity of the laboratory. For example, the oncology patient undergoing treatment may experience depression and may be medicated accordingly. Antidepressant and anticancer drug monitoring may help to correlate or differentiate between the clinical

response and side effects of anticancer and antidepressant medications. Since renal patients exhibit different drug-protein bindings, free drug levels would be a more reliable parameter for correlating with response. The metabolism of both pediatric and geriatric patients differs from that of the "normal" population. In addition, pediatric and neonatal patients, because of their age and size, might require special sampling protocol and/or assay procedure to avoid interference. Because of their lowered metabolism, dosage regimens of geriatric patient group should be adjusted according to clinical response and guided by plasma levels. In the case of obstetric patients, the fetal exposure to drugs in maternal blood and the infant's exposure to drugs present in breast milk also merit further clinical investigation.

Future LC instrumentation improvements may include standardization of fittings. The potential application of microbore and supercritical LC awaits further studies. In enhancing sample preparation robotics, sample preparation cartridges would be desirable. Also central to a successful LC assay is the column. Thus new columns such as the microbore, and column packings with various functionalities may offer enhanced selectivity. Chiral separation of optical isomers would be useful to correlate the plasma concentration of optically or pharmacologically active isomers to clinical response. Identification and sensitivity limits may be further improved by the more easily "manageable" MS, for example, mass selective or ion trap detectors for GC, the interface of LC to MS, the tandem MS-MS, and the coupling of FT-IR to GC and eventually to LC. Enhanced sensitivity may be also achieved using a laser light source.

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