Journal of Chromatography, 531 (1990) 3–50
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5405

Review

Analysis of drugs and other toxic substances in biological samples for pharmacokinetic studies

GEOFFREY W. PENG*

Drug Metabolism Research, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)

and

WIN L. CHIOU

Department of Pharmacodynamics, College of Pharmacy, University of Illinois, Chicago, IL 60680 (U.S.A.)

(First received March 16th, 1990; revised manuscript received April 28th, 1990)

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1. INTRODUCTION

Analysis of drugs and other toxic substances in biological samples (bioanalysis) has been undertaken for a variety of applications in medicine, toxicology, pharmacology, forensic science, environmental research, etc. For such applications in various disciplines, bioanalysis is of fundamental importance, as critical medical, environmental, technological and legal decisions are often made based on the analytical data. In addition, new discoveries and knowledge are also often derived from the analytical data of the concentrations of drugs and other analytes in biological samples, thus making many advances in these fields possible. For example, for some applications bioanalysis is undertaken to identify and/or quantify illicit drugs in biological samples for the detection of substance abuse [1–4]; to assay foreign substances in biological materials, sometimes *post mortem*, for evidence of a poison in toxicological and forensic cases [1,2,5]; to analyze trace elements and toxic substances in biological matrices in natural sciences and environmental research [6]; to monitor the concentrations of drugs and metabolites in tissues, blood plasma or serum and urine specimens for a better understanding of the pharmacology and toxicity of drugs [1,5,7]; and to apply therapeutic drug monitoring in the management of drug treatment in patients [1,5,8–14].

In a more recent development, bioanalysis has a special impact on the advances in pharmacokinetics. Pharmacokinetics and its application in clinical pharmacology have evolved and advanced rapidly in the past two decades parallel with the advances in bioanalytical technologies. The innovation and refinement in bioanalytical technologies, especially high-performance liquid chromatography (HPLC), gas chromatography (GC) and immunoassays, have substantially enhanced the specificity, sensitivity and accuracy in the measurement of drugs, metabolites and other toxic substances in small amounts of biological samples. These technologies have made the analysis of nanogram (ppb) or sub-nanogram levels of drugs and other toxic substances in complex matrices a routine procedure in most bioanalytical laboratories. The concentration–time data of drugs and metabolites in the biological samples, which have stimulated the rapid advances in the theory and application of pharmacokinetics, are the

basis for all pharmacokinetic endeavors. In addition to the management of drug therapy in patients through therapeutic drug monitoring [1,5,8–14], bioanalysis and pharmacokinetics together also play a vital role in the discovery and development of new drug products by facilitating the investigations in pre-clinical and clinical pharmacology, toxicology, clinical safety and efficacy, drug metabolism, bioavailability and formulation development [1]. The demands for pharmacokinetic analysis for various applications in turn has stimulated the rapid growth and expansion of industrial, clinical, academic and contract laboratories conducting analysis of drugs in biological samples in recent years.

The advances and applications of bioanalysis and pharmacokinetics have resulted in the publication of voluminous articles of research results in scientific journals. Many excellent books and reviews have also been written on the subjects of pharmacokinetics [15–21] and on the analysis of drugs in biological samples [1,10–12,22–29]. The purpose of this review, which emphasizes the aspect of bioanalysis in pharmacokinetics, is to summarize the basic concepts and principles of pharmacokinetics and to outline the potential pitfalls that are to be avoided and the requirements and special precautions that need attention in the analysis of biological samples to support pharmacokinetic studies. It is hoped that this summary can promote and facilitate the collaboration between the analysts and the pharmacokineticists and other health professionals in the management of drug therapy in patients, in research to elucidate the mechanisms of drug activity and toxicity and in the discovery and development of new drugs.

2 PHARMACOKINETICS

2.1. Overview

Pharmacokinetics is "the study of the time course of drugs in biological systems and the mathematical relationships required to develop models to represent the time-dependent changes in the concentrations (or amounts) of drugs in such systems to interpret the data" [15,16]. In a broad sense and for practical applications, drug metabolites, toxic compounds and other xenobiotics, and endogenous substances are also studied in pharmacokinetics, and the biological systems may include man and animals as well as isolated *ex vivo* organ perfusion systems [30–33] of liver, gastrointestinal tract, lung and kidney and other *in vitro* preparations. In the *ex vivo* and *in vitro* preparations, pharmacokinetics studies the changes in the concentrations of the drug and its metabolites as the drug in solution perfuses through the organ or the changes in the concentrations of the drug and its metabolites in the media over time In animal and in human, pharmacokinetics is concerned with the changes in the concentrations of the drug and its metabolites with time in the body fluids, tissues and *excreta.*

In pharmacokinetics, the drug concentration–time data are often analyzed in mathematical representations to reduce to some meaningful pharmacokinetic pa-

rameters. These parameters are consistent with the existing data and observations and are useful for prediction of the results of future experiments and for practical applications [15]. The ultimate goal of pharmacokinetics is to understand the relationship between the pharmacologic and toxic effects of drugs and their concentrations in the body fluids. Pharmacokinetics of a drug is related to the processes of its absorption, distribution, metabolism and excretion. Because of the complexity of these processes, studies using perfusion of isolated organs and other *in vitro* preparations are often conducted to obtain information which is important for the understanding of the pharmacokinetics of the drug *in vivo* in man and animals [32,33]. Although the discipline of pharmacokinetics is relatively new, pharmacokinetics in conjunction with pharmaceutics, drug metabolism, statistics and bioanalysis has made unparallel accomplishments in the management of drug therapy and in clinical pharmacology in the past two decades.

Since the onset, intensity and duration of the pharmacologic and toxic effects of drugs are often related to the concentrations of the drugs in the circulating blood or plasma in the body, pharmacokinetics is also closely related to the time course of the pharmacologic and toxic responses of the drugs. Such time course of the biological responses caused by drugs is termed pharmacodynamics. In a sense pharmacodynamics is concerned with the effect of the drugs on the biological systems while pharmacokinetics studies the effect of the biological systems on the drugs. Pharmacokinetics and pharmacodynamics together are powerful tools for the understanding of the mechanisms of drug actions and are the basis for therapeutic drug monitoring in management of drug therapy and other applications.

2.2. Drug administration and the fate of drugs in the body

While some drugs are used for topical or local effects, most drugs are absorbed into systemic circulation to exert their therapeutic effect. These systematically acting drugs are administered in various dosage forms *via* enteral routes or parenteral routes to gain access to the bloodstream for systemic distribution. The enteral routes of drug administration include buccal, or sublingual, gastric (oral), intestinal and rectal; the parenteral routes are intravenous, intramuscular, subcutaneous, intradermal, percutaneous, inhalation, intraarterial, intrathecal and vaginal [17]. Oral, intramuscular and intravenous routes are the most commonly used methods of drug administration, but recent research activities have also placed emphasis on the development of dosage forms for percutaneous absorption [34,35]. Drugs administered *via* gastric and intestinal routes are absorbed into portal veins, then pass through the liver before reaching general circulation. Drugs so administered may be metabolized during the first pass through the gastrointestinal tract and liver and thus are inactivated with the net result of a reduced bioavailability. This phenomenon is called first-pass metabolism. For most other routes of drug administration, drugs are either distributed before passing through the liver or are injected directly into the systemic circulation and

are not first-pass metabolized in the liver The lung is another site of potential first-pass metabolism and excretion [36].

After absorption, the drug undergoes the processes of distribution, metabolism and excretion. All of these processes affect the drug concentrations found in plasma, and inter-subject variations in drug levels are often observed, especially for highly first-pass metabolized drugs. Other factors such as stomach emptying and pH, presence of food, protein binding, disease state (hepatic and renal function impairment), age, body weight, metabolic capacity and drug interactions, also affect the drug concentration in plasma [17]. Distribution of the drug between different body fluids and tissues is usually reversible. At steady state or during the terminal exponential phase, the plasma drug concentrations often reflect the concentrations in other tissues including drug receptors. The absorbed drug may be eliminated as intact drug (excretion) and/or as metabolites (metabolism). Both the intact drug and metabolites are eliminated *via* biliary and/or renal excretion. Renally eliminated drugs are excreted by glomerular filtration and active tubular secretion, but some drugs may be reabsorbed *via* distal renal tubules. Biliary eliminated drugs are excreted in feces, but may also be reabsorbed from the intestine *via* the hepatoportal system into systemic circulation. In addition, biliary excreted drug metabolites such as glucuronides may be converted in the intestine to the intact drugs or other precursor metabolites and reabsorbed. These processes are termed enterohepatic recirculation.

Some drugs are extensively metabolized in the body and only metabolites are excreted. The liver is the primary organ for metabolism of drugs, but other tissues are also known to metabolize drugs and other xenobiotics. Drug metabolism reactions are carried out by various enzyme systems in the body and occur in two distinct phases [17,37]. Some drugs undergo both phase I and phase II metabolism, while others are metabolized by either phase I or phase II reactions. In phase I metabolism, drugs are biotransformed most commonly by oxidation but also reduction and hydrolysis reactions into metabolites. These reactions often render the metabolites suited for possible phase II reactions. Examples of oxidation reactions are aromatic and aliphatic hydroxylation, dealkylation, sulfoxidation, N-hydroxylation, oxidative deamination and dehydrogenation. Nitro reduction and azo reduction are the only major phase I reduction reactions. Phase I hydrolysis is catalyzed by the ubiquitous esterases which are active in blood, liver and other tissues. Phase I metabolites are usually pharmacologically inactive but for some drugs activation (trichloroethanol from chloral hydrate) and modification (morphine from codeine) of phamacological activity are observed. The phase II drug metabolism reactions include conjugation of phenols, alcohols, carboxylic acids, amines and halohydrocarbons with glucuronic acid, glycine and mercapturic acid, acetylation of amines and sulfonamides, sulfation of phenols, and methylation of phenols, amines and sulfhydryl compounds. The metabolites from phase II reactions tend to be more water-soluble and suited for renal and biliary excretion. However, exceptions such as acetylation of some sulfonamides which

reduce the water solubility of the metabolites are also known. Differences in metabolism of drugs often are important factors for the observed inter-species and inter-subject variation in pharmacokinetics and pharmacodynamics.

2.3. Pharmacokinetic approaches

Three different approaches to describe the pharmacokinetics of drugs have been adopted, namely, compartment modeling, physiological modeling and non-compartmental pharmacokinetics [15–18,38–41]. Compartment modeling, which depicts the body as being composed of one or more compartments, has been a most commonly used approach. These compartments do not represent actual physiological or anatomical entities of the body, but rather for each compartment the equilibrium of drug distribution is rapid among the components within the compartment, *i.e.* the components within the compartment are kinetically homogeneous. For compartment modeling, the drug concentration–time data are fit to compartment models using various computer programs which also estimate the pharmacokinetic parameters.

The simplest linear one-compartment model assumes that the drug entering the body is distributed and reaches equilibrium instantaneously throughout the body tissues and fluids. The drug concentrations in different tissues and fluids are not identical but only the equilibrium between tissues is rapid, and changes in concentrations in plasma reflect changes in drug concentration in other body fluids and tissues. The linear two-compartment model, which depicts the body as being composed of a central and a peripheral compartment, is used for drugs showing an initial distribution phase after administration The transfer of the drug between the central and peripheral compartments is associated with specific rate constants. The rate of drug transfer between compartments and the rate of elimination of drug from the body are proportional to its concentration, *i.e.* these processes follow first-order kinetics When the plasma drug concentration–time data are plotted on semilogarithmic scales, the drug exhibiting the one-compartment model behavior shows a single elimination phase or a mono-exponential decline of drug concentration with time, while the drug described by the two-compartment model exhibits a distribution or α phase and an elimination or β phase or a bi-exponential decline of drug concentration.

Although the pharmacokinetics based on compartmental modeling of many drugs has been intensively studied and applied in clinical practice for more than two decades, this approach is not without criticism and controversies. One such argument is the phenomenon of marked dependence of drug concentration on the blood sampling site. This phenomenon had led to the question of the scientific justification of basic compartmental modeling concepts [42–45]. Readers are advised to be aware of the recent developments and issues concerning the pharmacokinetic data analyses or conclusions based on the classical compartmental concepts.

The pharmacokinetic interpretation in physiological modeling uses known or assumed physiological values of blood flow-rate through various organs or tissues and the ratios of the concentration of the drug in tissues and blood to describe the transfer of drug. This approach has the advantage of being able to predict the movement of the drug in specific organs or tissues in both normal and pathological conditions. However, the associated mathematics can be very complex, and the model can only really be developed *in vitro* (perfusion of organs) or in animals. On the other hand, the clearance approach based on physiological modeling is relatively simple and practically very useful [16,38,44–46]. The non-compartmental approach is relatively recent and may not require either the assumption of a specific model or the fitting of the entire plasma drug concentration data into poly-exponential equations.

2.4. Pharmacokinetic parameters

2.4.1. Peak concentration, time to reach peak concentration, half-life and elimination rate constant

After intravenous bolus injection, the concentration of drug in plasma declines with time almost immediately and the highest concentration of the drug is observed within minutes after the injection [36,44]. After oral or intramuscular dosing, the drug concentration in plasma increases with time initially because of the rapid absorption of the drug into the bloodstream. As the amount of drug at the absorption site decreases, the rate of absorption also decreases and eventually reaches a point of maximal drug concentration in plasma when the rate of absorption of the drug into the general circulation is equal to its rate of distribution and elimination from the circulating plasma. After the maximum, the concentration of drug declines with time but the absorption continues at a lower rate until all the drug is removed from the absorption site. The first-pass retention of drug in the gastrointestinal tract and liver following absorption may significantly delay the appearance of drug in the general circulation [45,47]. Furthermore, recent studies showed that the initial ascending plasma drug concentration profile, including peak, time and concentration, may also be affected markedly by the sampling site [45,47].

From the data of plasma concentration of drug *versus* sampling time, the peak concentration (C_{max}) and time to reach peak concentration (T_{max}) are identified. The C_{max} increases as the dose and the rate of absorption increase and both the C_{max} and T_{max} can be dependent on the physicochemical and formulation factors of the drugs (solubility of the drug, disintegration and dissolution of solid dosage forms) as well as physiological factors of the subjects (stomach emptying and pH, active transport). The C_{max} and T_{max} together are often an indication of the rate of drug absorption. The terminal half-life ($T_{1/2}$) is a function of the elimination and distribution of the drug. The $T_{1/2}$ of the drug in circulating plasma can be esti-

mated from the elimination phase of the semilogarithmic plasma drug concentration–time plot. From the $T_{1/2}$ value, the elimination rate constant (β) can be calculated by the equation:

$$\beta = 0.693/T_{1/2}$$

Alternatively, from linear regression analysis of the logarithm of drug concentration *versus* time during the elimination phase, β is estimated from the slope of the linear regression line and $T_{1/2}$ can be calculated.

The half-life of a drug can be reliably estimated only after the cessation of the absorption and distribution phases. The half-life can vary for different drugs and for the same drug in different subjects, but is independent of the dose and the initial terminal-phase concentration in linear pharmacokinetics. Dependence of $T_{1/2}$ of a drug on its dose is an indication of non-linear kinetics and/or prolonged absorption because of enterohepatic cycling or slow release of drug from a solid dosage form.

The half-life or elimination rate constant of a drug can be an indication of the duration of its pharmacological activity and can be related to the frequency of dosing that is necessary to maintain therapeutic effect. Knowledge of the half-life of a drug also allows the pharmacokineticists and analysts to estimate the elapsed time after dosing that is necessary to collect and assay plasma and urine samples. It takes about four half-lives for 94% and six half-lives for 99% of the drug to be eliminated. Therefore, it is generally desirable to collect and assay plasma samples up to about four half-lives after drug administration in pharmacokinetic and bioavailability studies. However, for drugs with extensive distribution characteristics, the time needed for such a purpose could be much shorter [38,40].

2.4.2. Area under the curve, bioavailability, bioequivalence and dose proportionality

The area under the plasma concentration–time curve (AUC) is another important pharmacokinetic parameter that can be calculated from the plasma concentration–time data. The AUC can be estimated by linear and logarithmic trapezoidal rule [48] and is often corrected for the residual area extrapolating from the last sample with measured drug concentration to infinity (total AUC) To avoid potential error in the extrapolation of the area, it is generally important to have plasma samples collected and analyzed so that the area estimated by the trapezoidal rule accounts for at least 90% of the total AUC. AUC is an indication of the extent of drug absorption and is often used to evaluate the bioavailability of drugs in dosage forms.

Bioavailability (F) refers to the fraction of the dose of an administered drug that is absorbed into the general circulation and is often estimated by comparison of the AUC following different routes of dosing or following administration of drug in different formulations. The drug in intravenously injected dose is totally available, *i.e.* $F = 1$. Comparing a dose (D_t) of a drug in a test formulation and a

dose (D_r) of the same drug in a reference formulation in a cross-over study, the bioavailability (F) is estimated by the equation:

$$F = (AUC_t/D_t)/(AUC_r/D_r)$$

where AUC_t and AUC_r are AUC following administration of the drug in the test and reference formulations, respectively. When the reference formulation is given intravenously, F is the absolute bioavailability of the drug in the test formulation. The test formulation can also be a solution or a solid dosage form and administered by non-intravenous routes. If both the reference and the test formulations are administered orally, F is the relative bioavailability of the drug in the test formulation comparing to the reference formulation. These bioavailability studies are useful in evaluating the *in vivo* performance of tablet, capsule or other formulation compared to an intravenous dose or to an oral dose of the drug in solution or in other reference formulation.

The bioavailability studies are often conducted to compare the *in vivo* performance of two or more alternative solid formulations such as different tablet formulations. In these cases, the bioavailability is often called bioequivalence. Bioequivalence is concerned not only with the extent of drug absorption (AUC), but also with the rate of absorption (C_{max} and T_{max}). Therefore, a drug in two formulations can be equally bioavailable (same AUC) but not bioequivalent (different C_{max} and T_{max}), although bioavailability has also been defined as to require no significant differences in both the extent and the rate of absorption for the drug in two formulations to be equally bioavailable. The bioavailability of a drug over a dose range is also studied to evaluate the relationship between the AUC or C_{max} and the dose. These studies are termed dose proportionality studies. In linear pharmacokinetics, ideally the bioavailability (both AUC and C_{max}) of a drug is proportional to the dose of the drug.

2.4.3. Volume of distribution

To relate the amount of the drug (A) in the body and the concentration of the drug (C) in the analyzed sample, a proportionality constant is used as shown in the equation:

$$A = C\,V$$

and therefore, $V = A/C$. In this relationship, the proportionality constant V has the unit of volume and is called the apparent volume of distribution. The apparent volume of distribution is a function of time after dosing and the calculated value can also be markedly affected by the source of the blood or plasma sampled [44,45]. Different terms with different meanings such as initial volume of distribution, steady state volume of distribution and the distribution volume of the terminal phase are available [16,44,45]. The last two terms are most commonly used.

As a compartment in compartment modeling does not represent any real physiological entity in the body, so the volume of distribution of the compartment does not relate to a real physiological volume. For example, the volume of distribution in man may vary from a few liters for drugs that are highly plasma protein-bound but not distributed significantly into extravascular tissues to several hundred liters for drugs that are lipid-soluble and extensively bound to adipose tissues. The volume of distribution of a drug is useful for the estimation of the concentration that can be achieved after a given dose or the dose required to achieve a certain concentration in plasma. Drugs with a very large volume of distribution have low concentrations in plasma and the assay of these drugs is usually more challenging and difficult.

2.4.4. Total clearance, renal clearance and hepatic clearance

Clearance, a measure of the removal of drug from the body, is the volume of plasma or blood from which the drug is completely removed per unit of time. Since the liver and kidney are the major clearance organs for most drugs, the toal body clearance (Cl_T) is essentially the sum of hepatic clearance (Cl_H) and renal clearance (Cl_R) and can be calculated from the dose that is absorbed into general circulation ($F\,D$) divided by the total AUC.

$$Cl_T = Cl_H + Cl_R$$

$$Cl_T = (F\,D)/\text{AUC} \text{ or } Cl_T/F = D/\text{AUC}$$

where F is the bioavailability and D is the dose. For non-intravenous dose, $F \leq 1$ and Cl_T/F is the apparent total clearance. From the relationship $Cl_T = D/\text{AUC}$, it can be shown that Cl_T is related to the terminal-phase volume of distribution and the elimination rate constant or half-life:

$$Cl_T = V\,\beta = V\,(0.693/T_{1/2})$$

Clearance is influenced by hepatic and renal function and blood flow, urine flow-rate and pH, and age. The concept of clearance is useful in the evaluation of drug disposition and in clinical drug therapy as it may relate to both the volume of distribution and the half-life.

For estimation of renal clearance, the amount of intact drug excreted in urine during each sample collection interval is measured to calculate the urinary excretion rate and the cumulative amount of intact drug excreted. For the drug given intravenously or orally and completely available ($F = 1$), the renal clearance is the total clearance times the fraction (f) of dose excreted as unchanged drug in urine.

$$Cl_R = f\,Cl_T$$

When the fraction of the drug that is absorbed is not known, renal clearance is estimated from the slope of the linear regression of the urinary excretion rate *versus* the plasma concentration of the drug at the midpoint of the urine collection interval or from the slope of linear regression of the cumulative amount of drug recovered in urine *versus* the cumulative AUC. Hepatic clearance is usually estimated by the difference between the total clearance and renal clearance. Recent development has shown that, in theory, only the systemic arterial plasma drug concentration data should be used in the above analyses [44,45]. Otherwise serious errors may occur when there is marked arterial–venous difference.

2.4.5. Multiple-dose pharmacokinetics

When a drug is administered in multiple doses, the concentration in plasma increases until it reaches a plateau or steady state during which the peak steady state concentrations (C_{maxS}) as well as the trough steady state concentrations (C_{minS}) remain constant from dose to dose. At steady state, the area under the plasma concentration–time curve over one dosing interval τ ($AUC_{0-\tau}$) is identical to total AUC after a single dose. From this the mean steady state concentration (C_S) can be estimated by the equation:

$$C_S = \frac{AUC}{\tau} \quad \frac{FD}{V\beta\tau}$$

The concentration of a drug at steady state is dependent on the dose and dosing interval. Although for a long time it is generally accepted that the time required to reach steady state is dependent only on the half-life of the drug, for most drugs such required time is dependent on the characteristics of the area under concentration–time curve rather than strictly the terminal half-life [38,40].

2.4.6. Non-linear pharmacokinetics

Most drugs at therapeutic doses are eliminated by processes that are first order or apparent first order. However, for many drugs these processes, such as metabolism and active transport, are potentially saturable and some drugs (phenytoin and salicylates) exhibit saturable elimination or non-linear pharmacokinetic behavoir at therapeutic blood concentrations. These drugs show a linear plasma concentration–time plot and a curvilinear semi-logarithmic plasma concentration–time plot. The half-life of drugs with non-linear kinetics changes with their initial concentrations, in marked contrast with drugs obeying linear pharmacokinetics. Michaelis–Menten kinetics is often used to analyze the pharmacokinetics of drugs with non-linear characteristics.

3. BIOANALYSIS AND PHARMACOKINETICS

The bioanalytical method and its application to the assay of drug and its metabolites in biological samples are a prerequisite of all pharmacokinetic studies

[8,9,49–51]. It is perhaps not coincidental that the advances in pharmacokinetics paralleled the advances in bioanalytical technologies. In the past two decades, analytical chemists have made unparalleled advances in the physicochemical techniques such as GC, HPLC, thin-layer chromatography (TLC) and mass spectrometry (MS) and various combinations of these technologies in terms of sensitivity and selectivity. At the same time, important advances in radioimmunoassay (RIA), radioreceptor assay (RRA), enzyme immunoassay (EIA), and fluorescence immunoassay (FIA) have been contributed by biochemists and pharmacologists. It is also during these years that the theories and applications of pharmacokinetics have evolved and expanded

Although for the development of analytical methods and the assay of biological samples detailed knowledge of pharmacokinetics is not essential, an understanding of the basic principles of pharamcokinetics is helpful for the analysts in interpreting the assay results and in interacting more effectively with pharmacokineticists, clinicians and clinical pharmacists and other health professionals. For these colleagues, a detailed understanding of the analytical methodology is not necessary, but an appreciation of the complexity of the analysis of the biological samples and the awareness of the reliability of the data also are helpful in their interaction with the analysts, in making conclusions and decisions based the analytical data, and in the design of study protocols and the collection and handling of the biological samples.

Biological variations are usually large compared to the variability of analytical results, but the reliability of the assay results has always been a concern [11,52,53]. On the other hand, proper utilization of the drug level data has been questioned as it appears that, for example, therapeutic drug monitoring has been more successful in decreasing the pharmacokinetic variability than in insuring an appropriate pharmacodynamic response [54]. Clinicians and other health professionals are urged to adopt a decision analytic viewpoint when using drug concentration data and to involve in prospective studies and retrospective analyses of published data to establish critical data base [55].

A tolerable level of errors for analytical results and an adequate assay sensitivity (low limit of quantification) of the analytical methods may be difficult to define. A definition of assay precision based on clinically allowable errors in clinical chemistry has been proposed [56]. For pharmacokinetic studies, systemic approaches to evaluate this issue are lacking. Few cases with specific drugs comparing the pharmacokinetic parameters derived from the drug concentrations assayed by different analytical methods, which likely have different assay precision and accuracy, have been reported. For example, the pharmacokinetics of digoxin was compared using serum concentrations determined by RIA, fluorescence polarization immunoassay and affinity column-mediated immunoassay. The calculated pharmacokinetic parameters differed slightly, but the differences were not significant ($p > 0.05$), and it was concluded that the assay methods did not have an apparent effect on digoxin pharmacokinetics [57]. On the contrary, a

longer plasma half-life of about 8 h for procainamide was observed using an assay method that has a lower low limit of quantification to monitor plasma procainamide for 24 h after dosing. The previously reported half-life of procainamide of 2–4 h was based on the plasma concentrations measured up to 12 h after dosing [58–62]. This sampling time of 12 h was limited by the lowest concentration of procainamide in plasma that could be measured using the available methods. Although the differences in the estimated apparent clearance might not be of clinical significance [63], the longer half-life of procainamide is in agreement with the predicted half-life estimated from population volume of distribution and clearance [64], and is consistent with the underestimation of steady state drug accumulation based on the half-life of 2–4 h [65]. The longer half-life is also consistent with the apparently parallel decline of plasma concentrations of procainamide and its metabolite, N-acetylprocainamide, between 12 and 24 h, since the metabolism of procainamide to N-acetylprocainamide was reported to be reversible and the apparent half-life of N-acetylprocainamide was longer than the previously reported half-life of procainamide [62,66,67].

4 BIOANALYSIS REQUIREMENTS, PRECAUTIONS AND PITFALLS

4.1. Sample collection and storage

The biological samples most often collected for the determination of drug levels are plasma, serum and urine. Whole blood, saliva, cerebrospinal fluid and body tissues are also analyzed, but less frequently. Since the drug is not distributed uniformly in the body, its concentrations found in different biological samples will not be identical. Furthermore, drug concentrations in the same samples, *e.g.* blood, collected from different sites, may also be different The concentrations of insulin, lidocaine, alcohol, nitroglycerine, paraquat, procainamide and propranolol in arterial and venous blood obtained from various sampling sites are different. This phenomenon of sampling site-dependent drug concentration and its implications in pharmacokinetics, pharmacodynamics, toxicology and therapeutics has been reviewed recently [44,45]. For pharmacokinetics analysis, it is essential to collect the samples at the sites and times specified in the study protocol. If a delay in sample collection is inevitable, the exact sampling and dosing times should be noted. It is also essential to properly label the samples so that the study protocol number, type of sample, subject identification, treatment code or dose, and collection time and date can be clearly identified on the labels. When the samples cannot be analyzed immediately, the collected samples are usually stored frozen at $-20°C$ or $-70°C$ under the conditions that the drugs and metabolites are stable for the duration of the storage before the samples are analyzed [68,69]. This means that the information on the stability of the drugs and the metabolites in the biological samples to be analyzed is necessary in planning for the analysis of biological samples for pharmacokinetic studies.

The blood samples for drug analysis are usually drawn into evacuated blood collection tubes which are commercially available. These tubes come with different types of stoppers and may contain no anticoagulant for harvesting serum or a variety of anticoagulants for collecting plasma. The suitability of many types of these widely used commercial blood collection devices for pharmacokinetic studies and for therapeutic drug monitoring have been evaluated for several years. Because the concentrations of some drugs in plasma or serum samples were changed when exposed to certain collection devices, the type of collection tubes used has become an important variable to be considered for accurate determination and proper interpretation of drug concentration data. The devices for collection of blood samples and the potential interferences caused by the materials in the sampling devices to the analysis have been reviewed [70]. Specific attention directed towards the interactions between the drugs or their metabolites and the collection tube stoppers, between the drugs or metabolites and the separation gel, and the effect of anticoagulants on the measured concentrations of the drugs and metabolites will be discussed here.

The plasticizer tris(butoxyethyl) phosphate (TBEP) was previously used in the plastic caps of blood collection tubes. TBEP which leached out into the blood samples was found to displace basic drugs, such as imipramine, alprenolol, propranolol, lidocaine and quinidine, from their binding with α_1-acid glycoprotein. The released drugs were then re-equilibrated and distributed into the red blood cells causing their measured concentrations to be erroneously low in the separated plasma or serum samples [71–74]. Although the use of TBEP in blood collection devices has been discontinued and some new collection tubes were reported to have no effect on lidocaine concentrations when plasma samples were exposed to the stoppers [75], other chemicals and contaminants in the blood sampling devices may influence drug analysis and need to be evaluated [76].

More recently, some collection tubes have evolved to contain "serum separator gel" which upon centrifugation produced a physical barrier between serum and the coagulum for convenient separation of serum from the clot [77]. The concentrations of theophylline in samples processed in serum separator tubes containing polyester gel (SST) (Becton-Dickinson) or silicone gel (Corvac) (Monoject) were comparable to the concentrations in samples stored in other blood collection tubes [78–81]. Amiodarone and desethylamiodarone concentrations in serum prepared from blood samples with and without using separator gel (SST) were also similar [82]. On the other hand, the concentrations of imipramine, desipramine, amitriptyline and nortriptyline were decreased (up to about 16%, $p<0.01$) by the presence of the gel separator compared to serum and plasma samples not exposed to the separator gel [83–85].

The measured concentration of lidocaine in serum was reported to be decreased (6%, $p<0.01$) when the blood sample was collected in serum separator tubes (SST or Corvac) compared to blood sample not exposed to the separator gels [78,86]. The lidocaine depression was found to be independent of the blood

volume and the time of contact with the silicone gel [78]. However, another report indicated that the recovery of lidocaine from serum stored in tubes containing polyester gel may be concentration-dependent [79].

When blood samples collected and stored in tubes containing three different separator gels were compared, the concentrations of phenobarbital, phenytoin and carbamazepine showed time-dependent decrease up to about 10% in 24 h [86–88]. However, contact with the separator gel during sample processing was also reported to have no effect on the measured concentrations of phenytoin [78] and phenobarbital [82].

It is evident that the interactions between drugs and the blood collecting devices are different for individual drugs and some discrepancies in the reported interactions are also apparent. The decrease in drug concentrations when the sample was processed using separator gels was likely caused by binding of the drug to the gels [86]. Such binding of the drug is dependent on the surface properties of the gels and might be one of the reasons for the discrepancies in the results reported for phenytoin and phenobarbital [78,81,86–88] which were obtained under different conditions such as concentrations of the drugs, contact time and temperature. The apparent concentration-dependent recovery of lidocaine [79] is also consistent with the binding of the drug to the separator gel. The separator gel-caused depression in drug concentrations may be small for most drugs, *e.g.* $< 10\%$ for phenytoin over the concentration range 0.8–3.6 μg/ml [88]. In these cases, the tubes containing separator gel may be adequate for collection of samples for therapeutic drug monitoring, but may not be adequate and are not recommended for sampling for pharmacokinetic studies. Even minimizing contact time and using adequate sample volume may not obviate the binding effect and avoid the falsely low measured drug concentrations.

In addition to the adsorption to the serum separator gels, drug adsoption to glassware during storage and sample preparation, especially at verly low concentrations, could also be a potential problem for drug concentration determination. Significant adsorption of methotrexate onto glassware and syringe for HPLC assay has been reported [89]. Silanization to deactivate the glass surface [90] or use of alternative containers can often alleviate this problem.

4.2. Effect of anticoagulants

Plasma and serum samples are used most often for drug analysis for pharmacokinetic studies. It is frequently assumed that the drug concentrations in the simultaneously collected plasma and serum samples are identical and the two biofluids are interchangeable for the purpose of determination of the concentrations of drugs and metabolites. However, while such assumption may be correct for many drugs, exceptions are known. In addition, the processes by which the plasma and serum samples are prepared may affect the measured concentrations

of drugs. For example, the total concentrations of valproic acid in plasma samples with citrate anticoagulant were significantly lower (20–25%) than the total drug concentrations in serum or in plasma with heparin as anticoagulant, while the total and free valproic acid concentrations in serum and in heparinized plasma were not different [91]. The mean concentrations of amiodarone and desethylamiodarone in serum from patients were lower (about 10%) than the means in the simultaneously collected heparinized plasma samples [82]. Like many other drugs, the concentrations of tricyclic antidepressants were determined in both plasma and serum samples in literature reports [92,93]. Some of the serum and plasma concentration data of the tricyclic antidepressants are inconsistent. For example, the mean concentrations of imipramine in heparinized plasma samples were reported to be 32% higher [94] and 5% lower [82] than in serum, while the concentrations of desipramine, amitriptyline and nortriptyline were not significantly different in plasma and in serum samples. Some reports are confusing as to which biofluids were actually analyzed [95,96].

The effect of commonly used anticoagulants on the measured concentrations of drugs in plasma has not been investigated in detail. Such effect on the measured concentrations of endogenous trace elements has been demonstrated [97,98]. Heparin is known to activate lipoprotein lipase, and the concentrations of free fatty acids in heparinized human plasma were reported to be higher (up to 60%) than the concentrations in serum or in plasma prepared with EDTA as anticoagulant [99]. Comparison of the measured concentrations of selected trace elements, vitamins, lipids and proteins demonstrated that the concentrations of all these clinical–chemical analytes in citrated plasma were significantly lower ($p < 0.05$) than in heparinized plasma [100]. The lowering of the measured concentrations of these analytes were due to the hyperosmolar citrate anticoagulant in the blood samples in which the hyperosmolarity of the citrate induced leakage of intracellular water from red blood cells and caused a significant dilution of the citrated plasma [97,100].

Although the effect of citrate as an anticoagulant on the analysis of drug and metabolite in plasma has not been systematically studied, the lowering of the concentrations of valproic acid in citrated plasma samples [91] is likely due to the effect of the dilution of the plasma caused by the hyperosmolar citrate anticoagulant. The increase in free fatty acid concentrations in stored plasma could, in addition to enzymatic degradation, be associated with the reduced recovery by solid-phase extraction of spiked edrophonium and pyridostigmine from frozen and stored plasma compared to fresh plasma [101].

4.3. Preparation of biological samples for analysis

Biological samples are very complex multi-component mixtures. Drugs and other toxic substances are often present in these samples as minor components. Selective isolation of the analytes from the samples and preconcentration proce-

dures are often necessary prior to separation and measurement. Solvent extraction, solid-phase extraction, on-line solid-phase extraction using the column-switching technique and protein precipitation are used to isolate and enrich drug and other toxic substances from the biological samples [102]. The basic drug can be extracted into the organic solvent from the alkalinized samples in which the high pH suppresses the ionization of drugs and renders their partition into the organic solvent favorable. Occasionally, the basic drug extracted in the solvent can be extracted into an acidic aqueous solution which is then basified and re-extracted with solvent for chromatography. Sometimes, the biological samples are acidified and extracted with an appropriate solvent to remove interferents and then basified, and the basic drug is extracted with an organic solvent for chromatographic separation. Acidic drugs can likewise be extracted, taking advantage of their pH ionization and partitioning characteristics. Ionizable drugs have also been isolated by ion-pairing extractions [103–105]. Extraction procedures should be optimized to obtain high recovery of the drugs and metabolites as exemplified by the assay of amiodarone and its metabolite N-desethylamiodarone in serum by an HPLC method [106] In this assay, the hexane extraction gave good recovery (82%) of the parent drug, but poor recovery (22%) of the more polar metabolite while a mixture of methylene chloride and methanol was found to extract both the parent drug and the metabolite efficiently (>92%).

Recently, many different types of cartridges for solid-phase extraction are commercially available. By proper selection of these cartridges and the elution solvents, drugs and toxic substances of a wide range of physicochemical properties can be isolated from the biological samples to facilitate chromatographic separation. Both off-line [106–108] and column switching on-line solid-phase extraction procedures [109] are increasingly used for sample preparation because of the convenience and versatility of these procedures. Some good examples of solid-phase extraction procedures are the HPLC assays of chlorpromazine and its thirteen metabolites [110] and of extremely low levels (pg/ml) of bopindolol in plasma [111]. On-line solid-phase extraction procedures were utilized in the analysis of diltiazem [112], dihydroergotamine [113] and dextromethorphan and its metabolites [114]. Alternative to the pre-packed cartridges, adsorption of catecholamines on alumina was successfully used to isolate the catecholamines from plasma samples for HPLC analysis [115].

Immunoaffinity purification of drugs and metabolites has been utilized to isolate these analytes from biological samples for separation and quantification. Plasma samples containing *erythro*-9-(2-hydroxy-3-nonyl)hypoxanthine were incubated with antiserum to form drug–antibody complexes which were precipitated with ammonium sulfate, and the drug was recovered in ethanol for HPLC analysis [116]. Antibodies immobilized on Sepharose and silica gel were used to isolate thromboxane B_2 and 2,3-dinor-thromboxane B_2 [117,118] and 6-keto-prostaglandin $F_{2\alpha}$ [119] in urine or plasma for GC–MS analysis Monoclonal antibodies have also been used for isolation of chloramphenicol in aqueous extract of swine muscle tissues for measurement by HPLC [120].

Various approaches have been investigated to simplify the handling and preparation of biological samples. Separation of drugs and metabolites from plasma proteins by ultrafiltration and by precipitation using methanol, acetonitrile, acetone, trichloroacetic acid, etc. have been used to obtain protein-free samples for HPLC analysis [102,121,122]. Ibuprofen and furosemide in plasma were analyzed by injection of the supernatant after mixing the samples with acetonitrile and methanol [121,122] for chromatography. Urine samples of ibuprofen were chromatographed by direct injection after hydrolysis of glucuronide and pH adjustment [121].

Recently, direct injection of serum, plasma, urine and whole blood samples for HPLC analysis has been investigated for assay of drugs and metabolites. Direct injection procedures of serum samples on the HPLC column for the analysis of drugs have been reviewed [123]. These approaches include the use of a guard column, wide-pore column, internal surface reversed-phase column, column-switching and micellar chromatography [123]. By direct injection of the biological fluids, the sample handling is greatly simplified, the use of internal standard is not as important as other sample preparation procedures, the speed of analysis is enhanced, and the cost may be reduced. On the other hand, shortening of column life, requirement of special columns and the complexity of the instrument for column switching are some of the disadvantages of the direct injection procedures. The direct injection procedures are applicable in most cases only for samples containing relatively high concentrations of the drugs, unless the analytes are enriched by some means. Column-switching procedures and laboratory robots have been used successfully to fully automate the preparation of the samples for HPLC analysis [124–126].

Among the procedures of direct injection of biological samples for HPLC analysis, the direct injection of whole blood samples and the development and application of specialized columns are of most interest. Direct injection of whole blood samples was investigated using column-switching procedures [127,129]. By these procedures, the carbamazepine concentration in rabbit blood cells was found to be 1.1 to 1.3 times higher than in plasma [127]; procainamide was metabolized to N-acetylprocainamide in the blood cells [128]; and chlorpromazine bound to cytomembranes was measured by the differences in the concentration found in whole blood and in the supernatant of hemolyzed whole blood samples [129]. Different approaches were undertaken in the design of column packing materials for direct injection of plasma and serum samples. Shielded hydrophobic phase [130], small-pore reversed-phase [131] and internal surface reversed-phase [132] columns elute serum proteins in the excluded volumes and allow small molecules (most drugs and metabolites) to interact with the stationary phase for separation. A wide-pore reversed-phase column elutes small molecules while selectively allows proteins to permeate through the large pores without being hindered [123]. Direct injection of serum samples containing phenobarbital and carbamazepine using a shielded reversed-phase column has

been evaluated [130]. Theophylline and carbamazepine in serum samples were separated by wide-pore reversed-phase columns [123,133]. Propofol in plasma samples [134] and peptide toxins produced by cyanobacteria [135] were analyzed by internal surface reversed-phase columns. These columns were also utilized in tandem with cyano column for analysis of 2′,3′-dideoxycytitidine and 3′-azido-3′-deoxythymidine (AZT) in plasma [136]. In another application, warfarin in serum was separated by direct injection of the samples onto an internal surface reversed-phase column which was coupled with a bovine serum albumin column to analyze the enantiomers of the drug [137].

4.4. Stability of drugs in biological samples

Many drugs and their metabolites are relatively unstable and may degrade in the biological samples during the collection and processing of the fresh samples and the subsequent storage, preparation and analysis of the samples. Factors affecting the stability of drugs in biological samples are many and should be evaluated based on each individual drug. Systemic approaches based on statistical treatment of data for the determination of the stability of drugs in biological samples have been developed [138,139]. In most pharmacokinetic studies the biological samples collected are seldom analyzed immediately after collection, but are stored for later analysis. Long-term storage enhances the probability of degradation of the drugs and metabolites in the biological samples. The analysis of biological samples should be completed before the degradation of the drugs occurs. In addition, degradation products unrelated to drug metabolism which are generated during the preparation of the samples may be mistaken as metabolites [140]. Care must be taken to assure that the measured drug concentrations are meaningful and not interfered with by sample degradation and other artifacts due to the procedures for the collection, processing, storage and analysis of the samples.

Blood specimens collected from man and animals are often kept at ambient temperature or refrigerated for a variable length of time before centrifugation to prepare plasma or serum samples. In order to more accurately measure the *in vivo* plasma drug concentration, it is sometimes important to centrifuge the blood sample immediately after collection. This is because the redistribution of drug by diffusion across the erythrocyte membrane may occur under different circumstances for some drugs such as procainamide [141], methotrexate [142] and possibly doxorubicin [143], and the redistribution may cause a significant overestimation or underestimation of the true plasma concentrations. Such a phenomenon has been called "blood storage effect" [141–143]. In addition, the conditions for processing whole blood samples can also affect the measured concentrations of drug or other analytes [144,145]. The temperature and the time of the contact of serum with cell clot were reported to make significant differences (mean percentage change $>50\%$) in the measured concentrations of creatinine,

glucose, inorganic phosphate and potassium, and the activities of alanine and aspartate aminotransferases [145]. For collection of samples containing unstable analytes, using a prechilled container, keeping the sample over ice, and centrifuging at 4°C tend to minimize the decomposition of the analytes. For example, these conditions of low temperature were found to be more favorable than the use of enzyme inhibitors for preserving immunoreactive neuropeptides and other regulatory peptides [146].

Often the biological samples need to be transported to other locations for assay. This may present special difficulties for drugs and other analytes that are unstable. Human plasma and serum samples frozen in liquid nitrogen for long distance air transport were adequate for determination of the concentrations of catecholamines, but $>60\%$ decrease in the measured catecholamine concentrations was observed in the lyophilized plasma or serum transported at ambient temperature [147]. For renin activity, aldosterone, prolactin, cortisol, vasopressin, growth hormone and atrial natriuretic peptide, freezing of samples in liquid nitrogen as well as lyophilization is adequate in terms of stability in storage and during transport [147].

Drug and metabolites may be degraded chemically [140,148], photochemically [149] or enzymatically [132,150,151] in biological samples. The degradation of biogenic amines is an example of chemical instability. It was reported that light and air were detrimental to the stability of indoles in brain tissues [148]. In the absence of light, their stability was dependent on temperature and the presence of air; however, in the absence of air, light and temperature had little effect. Addition of antioxidants appeared to enhance the stability of these biogenic amines.

Clonazepam in control serum samples in glass or plastic tubes were degraded by UV light and direct and indirect sunlight after exposure of 2 h. The concentration of clonazepam was reduced to less than 50% of its initial value [149] This photochemical degradation of clonazepam was not observed under the ordinary indoor lighting conditions. Although such experiments of UV irradiation and direct sunlight exposure were conducted under artificial conditions, the indirect sunlight through double-glassed window could mimic actual laboratory conditions if the sample tubes are left near a window. For drug or other analytes that are light-sensitive, the samples should be protected from light or their exposure to light should be minimized.

Enzymatic degradation of drugs catalyzed by enzymes in sample matrices can occur and affect the measured concentrations of the analytes. Many enzymes such as cytochrome P450, esterases and lipases retain their activities *in vitro* in tissue sample homogenate and in plasma samples. It is therefore not surprising that chloramphenicol was metabolized in tissue samples *in vitro* [150]. Chloramphenicol was rapidly metabolized *in vivo* involving oxidation catalyzed by cytochrome P450 system followed by glucuronic acid conjugation. Cytochrome P450 is present at high activity in the liver and at lower activity in other tissues [151]. Pretreatment of the tissue samples with piperonyl butoxide, a potent inhibitor of

P450, minimized the loss of chloramphenicol caused by *in vitro* metabolism [150]. The ubiquitous esterases can hydrolyze esterified metabolites to parent drugs or precursor metabolites and ester prodrugs to active drugs in the sample. On the other hand, procainamide was metabolized via acetyltransferase *in vitro* [128,141]. Lipases can change the lipid composition in the samples and thus can modify protein binding of drugs and the assay extraction recovery [102].

In the biological samples, some metabolites of drugs may convert to parent drugs or other metabolites and thus may result in falsely high concentrations of the measured parent drugs or the metabolite precursors. This could happen potentially more frequently in whole blood samples than in plasma or serum because red blood cells contain glutathione, cysteine residues of hemoglobin and other reductive systems [152–154] that can cause the formation of the artifacts. Examples are the reported interconversion between imipramine and its N-oxide in whole blood [155] and acetylation of procainamide by N-acetyltransferase in blood cells [128]. When added to whole blood lysed with alkali, chlorpromazine was partially (10–14%) oxidized to chlorpromazine sulfoxide, and chlorpromazine N-oxide added to the lysed blood was rapidly reduced to chlorpromazine which was in turn partially oxidized to its sulfoxide [156]. In another study, chlorpromazine N-oxide was reduced to chlorpromazine in plasma basified with sodium hydroxide, but this conversion was not observed in plasma basified with sodium carbonate [157]. Because of such conversions, the apparent concentrations of chlorpromazine in plasma from patients were elevated to as much as more than 300% when the plasma samples were basified with sodium hydroxide compared to sodium carbonate before extraction with organic solvent for chromatographic analysis [157].

The concentration of 5-fluorouracil in whole blood kept at ambient temperature was decreased by 94% in 24 h while the concentration reduction was by 52% in plasma samples under the same conditions [158]. When the blood and plasma samples were maintained on ice, the loss of 5-fluorouracil was only 30 and 10% for the whole blood and plasma samples, respectively, over 24 h. 5-Fluorouracil is eliminated from the body *via* the normal catabolic pathways for uracil and thymidine. The observation that uracil added to whole blood or plasma samples competitively inhibited the breakdown of 5-fluorouracil suggests that the drug was metabolized by the same enzymatic catabolism *in vivo* and *in vitro* [158].

Many phase II metabolites of drugs such as glucuronides and sulfate conjugates are labile and subject to hydrolysis. The products of the hydrolysis of these metabolites are often the parent drugs or phase I metabolites, thus resulting in falsely high concentrations of the parent drugs or their phase I metabolites [159]. In addition, the acyl glucuronides of many drugs or metabolites containing carboxylic acid moieties undergo facile rearrangement (*via* acyl migration) to further complicate the analysis of these drugs in biological samples and the evaluation of their pharmacokinetics and metabolism [160]. These complications are exemplified by the recent reports of the HPLC analysis of diflunisal [161,162]. Diflunisal

was metabolized to phenolic and acyl glucuronides in animal and in man [163]. More recently, a sulfate conjugate was identified in human urine [164]. Among these metabolites of diflunisal, the phenolic glucuronide is relatively stable and non-reactive, but the sulfate conjugate is rapidly hydrolyzed under acidic pH and the acyl glucuronide is also labile and hydrolyzed under netural and slightly alkaline pH. In addition, the diflunisal acyl glucuronide also undergoes acyl migration and mutarotation to form isomers and solvolysis and transesterification to form diflunisal methyl ester in presence of methanol [162].

Based on the reactivity of these metabolites, a non-extractive and non-concentrative direct analysis by working at a pH of approximately 4.5 was necessary for the accurate determination of the concentrations of diflunisal in plasma and urine samples. These conditions are also necessary for the simultaneous determination of diflusinal and its reactive and non-reactive metabolites. Many assay methods developed earlier [165–167] without this complete information of metabolism and the stability of the metabolites tend to overestimate the concentrations of diflunisal due to the conversion of the labile metabolites to the parent drug.

In addition to the potential of degradation of drugs in biological samples, procedures for collection, processing and preparation of biological samples that displace drugs from their binding to macromolecules and cause redistribution of drugs between plasma and red blood cells can affect the measured concentrations of drugs in plasma [141–143]. Dilution of plasma with Tris buffer was shown to significantly increase the concentration of catecholamines measured by radioenzymatic assay [168]. This elevation of catecholamines in plasma was attributed to the displacement, caused by Tris buffer, of catecholamines and conjugated catecholamines from some unidentified low-molecular-mass component of plasma. Hemolysis was reported to reduce analytical recovery of catecholamines by radioenzymatic assay [169], but this effect was not confirmed by analysis using HPLC with electrochemical detection [170].

4.5. Reference standards

All quantitative chromatographic analyses of drugs and metabolites in biological samples are based on the comparison of the detection signals generated by the analytes in the samples with the corresponding signals from the calibration standards. The calibration standard samples are prepared by adding known amounts of the reference standard compounds to the blank biological matrices. Therefore, the purity of these reference standards is of critical importance [171–173]. However, it appears that the purity of the reference standards has not attracted much attention of most analysts. Usually the information regarding the purity of the reference standards is not reported in the literature and one has to assume these reference standards are pure for the purpose of the assay. Special problems can arise with regard to the reference standards in analysis of antibiotics, peptides and other biological and recombinant products which may contain more than one

active compound or variable amounts of impurities originating from the production processes and from chemical and enzymatic degradation. For analysis of enantiomers of drugs, both chemical and optical purity of the reference standards are important.

4.6. Internal standard

The internal standard technique has been widely used in chromatographic analysis of drugs in biological samples. Prior to analysis, an accurate amount of internal standard is added to the samples which are then prepared and chromatographed. By this approach, errors in sample preparation and analytical measurement can be reduced since any loss of the drug in the samples through the analytical processes is compensated for by the similar or proportional loss of the internal standard. The ratio of the peak height (or area) of the compounds to that of the internal standard is used to establish the calibration curves for calculation of the drug concentrations in the samples.

The precise measurement and transfer of a small volume of reagents and samples during sample preparation or for the injection of the prepared sample for chromatography used to be difficult to achieve. The use of an internal standard in the assay can correct the imprecision and improve the analytical accuracy. Such precision of volume measurement has been substantially improved with modern analytical instruments. For example, the precision of sample injection using full loop filling can be as precise as 0.05% relative standard deviation. Thus, excellent precision is obtainable without the use of internal standard. The question has therefore been raised as to the necessity or usefulness of the internal standard technique in the analysis of drugs [174]. The answer to this question really depends on the choice of the internal standard [175]. With a properly selected internal standard, the errors arising from a loss of sample volume, changes in column efficiency or detector performance can be compensated. Thus, the internal standard technique still offers better precision. On the other hand, an improper internal standard can impair rather than improve the assay precision [175].

Guidelines for application of the internal standard techniques in the assay of drugs in biological samples have been delineated [26,174,175]. Ideally, an internal standard should be stable, have physicochemical properties close to those of the compounds being analyzed and elute near the peak of the compound but be completely resolved from the peak of the compound in the chromatographic separation. The internal standard should be added to the samples as early as possible during the analysis steps. The peak height (or area) of the internal standard should be similar to that of the drug, and in cases where a range of the concentrations of the drug is anticipated, as in samples from pharmacokinetic studies, the peak height (or area) of the internal standard should approximate that of the samples at mid-range of the concentrations. If the drug being analyzed

is derivatized before chromatography, the internal standard should be capable of undergoing the similar derivatization. When a mass spectrometer is used with chromatography, a stable isotope-labeled form of the drug is the most ideal internal standard.

4.7. Validation of analytical methods

For the application of the analysis of biological samples, validation is an important task since the analytical results are often used in making decisions in the diagnosis and the treatment of diseases in patients, or in vital clinical and non-clinical pharmacology, toxicology and pharmacokinetic studies and environmental research. In addition, governmental regulatory agencies overseeing environmental, health and pharmaceutical industries are also requiring strict controls and standards on the analytical methods for environmental and biological samples. The analysts have the responsibility not only to develop analytical methods to measure very low levels of drugs and other toxic substances in complex sample matrices, but also to prove that the results of the analysis are reliable and indubitable.

The demand and broad application of the analysis of drugs and toxic substances in biological samples have stimulated intensive research activities which have resulted in continuous publication of a large number of analytical methods in literature in recent years. Adaptation of these published methods, however, sometimes requires modifications and improvements to make the methods suitable for the laboratories conducting the assays. This is often also true when an assay method is transferred to other laboratories or is performed by another analyst. While some difficulties in duplicating or transferring analytical methods have been noted and tolerated, a major deficiency in reporting the analytical quality control data in clinical pharmacological literature has been identified [176]. All these point to the critical need of the validation of analytical methodologies. In general, the validation of analytical methods requires the demonstration of the specificity, sensitivity, calibration linearity, extraction recovery, precision and accuracy of the methods. Some other aspects usually not considered as part of method validation but which need to be considered to insure the reliability of the analytical data include sample collection and storage, reference standard, internal standard, quality control, assay method ruggedness and data acquisition and management [49,177–181].

4.7.1. Calibration and linearity

Calibration is one of the most important steps in chemical analysis. Without a good calibration procedure, precision and accuracy cannot be obtained [182]. For analysis of drugs in biological samples, drug-free specimens of the same biological fluids supplemented with accurately measured amounts of the drug of high purity (reference standard) and blank samples are employed as calibration stan-

dards. A solution of reference standard compound in water, solvent or other biological matrices generally cannot be used for calibration purpose, since the sample matrices affect the recovery and separation of drugs in the analysis. A preferred method for the preparation of the calibration standards (and seeded control samples) is to add small volumes of solutions of drug (reference standard) and internal standard to the blank biological samples. The calibration standards, samples and seeded control samples should contain the same volume of the added solvent due to the addition of the stock solutions of the drug and/or internal standard to assure good and consistent recovery. When acetonitrile, methanol or other solvents are used to prepare the stock solutions of the reference standard and internal standard, the volume added to the calibration standards, seeded controls and samples should be small, preferably $< 5\%$ of the sample volume, except when these solvents are used to precipitate plasma proteins. These samples should be properly mixed before further sample work up. Sometimes, aliquots of the reference drug standard and internal standard solutions are evaporated to dryness and then the blank biological samples are added to prepare these samples. In these cases, care should be taken to assure that the drug and internal standard are dissolved in the blank sample matrices The blank samples are included in the calibration samples to assure that no interferents which are coeluted with the drug or the internal standard are present in the samples or introduced during the preparation of the samples. The concentrations of the drug and metabolites in these calibration standards should bracket the expected range of the concentrations of the analytes in the samples Samples with concentrations found higher than the upper limit of the concentration of the calibration standards should be re-analyzed by dilution of the samples, or a different set of calibration standards containing higher concentrations of the drug should be used to estimate the unknown concentrations.

For most chromatographic analytical techniques, a first-order (linear) relationship is observed between the detector response (y)and concentration (x) of the analyte in the samples in the form of linear calibration curve obtained by least-squares linear regression procedures [182–184]:

$$y = a\,x + b$$

where a is the slope and b is the intercept of the calibration curve. Ideally, a should be reproducible from assay run to assay run and b should not be significantly different from zero. The linear regression should also have a high correlation coefficient ($r > 0.99$). The correlation coefficient has an ideal value of unity but is affected by the range and distribution of the concentrations of the drug in the calibration standards. This first-order linear relationship between the analytical signal and drug concentration may not hold true over a wide dynamic range of concentrations Therefore, alternative models and statistical procedures have been used to establish calibration curves to improve assay precision and accuracy.

Logarithmic transformation of data, using variance as a weighting factor, utilizing multiple linear calibration curves within a concentration range and employing least-squares best-fit of higher-order polynomial are some of the possible alternatives [182]. Other procedures employed include the use of a confidence interval for calibration curves to estimate the confidence interval of the concentrations of analyte in the samples [185,186] and Bayesian calibration [187].

Among these different approaches for the calibration in the analysis of drugs in biological samples, the least-squares linear regression is most often used. In cases where apparent non-linearity is encountered, dilution of samples containing high concentrations of drugs and use of multiple linear calibration curves can often overcome the difficulties and generate data with good precision and accuracy. Once the linear relationship between the detector response and the drug concentration in the samples has been established over a range of concentrations, a method of response factor can be used instead of the conventional least-squares best-fit linear equation. Response factor is the mean of peak height (or area) ratio divided by the concentration of drug in the calibration standards, and is used to calculate the unknown concentration of the drug in the samples. With linear regression of the calibration data, often times the linearity at low concentrations may be poor, but good correlation is still obtained because of the dominant contribution from the high concentration calibration samples in the linear regression analysis. In these cases, the use of a response factor for a certain range of concentrations for calibration may offer some advantages. The use of non-linear calibration curves for analysis has been discussed [188].

When an assay method is performed repeatedly to analyze a high volume of samples, the instability of the calibration curves or an apparent change in response factor often indicates some conditions of the assay are drifting, are no longer stable and need to be evaluated. The reason for the instability of the calibration curves can include variation of extraction procedures or recovery, deterioration of the column efficiency or detection system [189]. When the biological sample matrices interact with the drug to be analyzed, special problems can be encountered. For example, in the analysis of halothane in blood samples by a head-space GC method, the equilibrium of halothane between the gas phase and the sample was dependent on the lipoid content of the blood samples. A method of standard addition gave assay results that had better precision and accuracy than the traditional method using calibration standards to establish calibration curves [190].

4.7.2. Selectivity, specificity and interference

The specificity of an assay of a drug means that the endogenous substances, drug metabolites and other drugs present in the samples do not interfere with the determination of the concentration of the drug which is often a minor component in the samples. An extensive review on the interferences in the assays of commonly monitored drugs, digoxin, gentamicin, phenobarbital, phenytoin, procain-

amide, quinidine, salicylates and theophylline, in plasma has been published [191]. Sample work-up procedures, chromatographic separation techniques and selective detection all contribute to the assay specificity.

The specificity of a chromatographic assay depends largely on the chromatographic separation. The mechanism, theory and techniques of chromatographic separation have been amply reviewed [192–196] and will not be further discussed here. The detection systems commonly used in chromatographic analysis, such as UV absorbance, fluorescence and electrochemical detectors in HPLC and electron-capture and element-specific detectors in GC, also contribute to the assay specificity, although in general to a limited extent. GC–MS and LC–MS have increasingly been used for analysis of drugs and metabolites in biological samples, despite the complexity and the cost of the instrumentation. A mass spectrometer, when interfaced with the chromatographic system, provides the most reliable assay specificity.

Since the introduction of multi-wavelength absorbance detectors for monitoring HPLC separations, various techniques have been developed to identify and assess the purity of chromatographic peaks. These include three-dimensional wavelength–time–absorbance plots [197], spectral overlays [198,199], higher derivative transformation of spectra [200,201], isoabsorbance plots [202,203], peak purity parameters [204,205], absorbance ratios [206–208] and modeling for assessing interference [209]. These techniques in general require high concentrations of drugs and interferents in the samples to be chromatographed and the known spectral characteristics of these compounds under investigation [210]. These techniques are useful to assess the chromatographic peak purity but have not been routinely applied to quantify analytes in partially resolved chromatographic separation for sample analysis.

4.7.3. Sensitivity, limit of quantification and limit of detection

These terms have been defined in different ways [211–222], thus giving rise to a great deal of confusion. The limit of detection has been defined as the lowest concentration or quantity of an analyte that an analytical method can detect with reasonable certainty [211] or can reliably detect [212]. These descriptions allow considerable freedom to define the limit of detection based on the definition of "reasonable certainty" and "reliably detect" In the literature of analytical chemistry, the commonly used definition is that the limit of detection is the concentration of an analyte which gives a measured signal equal to the mean blank signal plus three times the standard deviation of the blank signal [182,183]. The concentration of an analyte which gives a measured signal equal to the mean blank signal plus a higher multiple (*e.g.* ten times) of the standard deviation of the blank is reported as the limit of quantification [49,182,183,185] The y-intercept of a linear calibration curve has been used as a more accurate measure of the blank signal to define the limit of detection and the limit of quantification [185,223]. These terms have also been defined as analyte signal-to-background noise (S/N)

ratios. For example, an S/N ratio of 3 has been used to define the detection limit and a higher S/N ratio as the limit of quantification. The limit of detection and limit of quantification as defined are often not very stable characteristics of an analytical method, since the blank signal and the signal generated by the very low concentrations of an analyte are often dependent on the conditions of the analysis such as temperature, purity of reagent, sample matrices and instrument system. The assay sensitivity is the measured analyte signal per unit concentration of the analyte and thus is the slope of the linear calibration curve [182,183]. However, the limit of detection or quantification has often been reported as the assay sensitivity.

The limit of quantification is more relevant than the limit of detection for the analysis of drugs in biological samples for pharmacokinetic studies and therapeutic drug monitoring. For these applications, the limit of quantification can be defined as the lowest drug concentration in biological samples that can be assayed with a desired level of precision and accuracy. Analytical methods for pharmacokinetic studies should have an adequate low limit of quantification to measure the low drug concentrations in biological samples necessary for pharmacokinetic evaluation.

4.7.4. Precision and accuracy

Precision and accuracy determine the error of analysis and are the most important criteria for judging the performance of an analytical method. Both terms have also been defined differently [182,183,211,224–236]. Normally, precision refers to the variation of scatter of the measurements about the mean value. Some analysts use the terms reproducibility and repeatability instead to refer to the scattering of the assay results obtained by the same method but under different and same conditions (*i.e.* operator, laboratory, instrument, time), respectively. For analysis of drug and other substances in biological samples for pharmacokinetic evalutation, precision and accuracy are most often estimated by analysis of replicate seeded control samples at several concentration levels, preferably three or more, over the expected concentration range of the samples. The precision of the analysis is estimated as the relative standard deviation (coefficient of variation) of the measured concentrations of replicate samples and the accuracy is estimated as the percent differences (bias) between the mean values and the true or known concentrations. When the analysis of the seeded control samples are carried out in the same assay run or on the same day the precision data are reported as within-day precision as compared to between-day precision for which the analysis is performed on different days. The criteria for acceptable precision and accuracy of the assay results cannot be generalized because both precision and accurancy depend on the concentrations of the analytes being evaluated [227] and the acceptable criteria depend on the purpose of the analysis. In the authors' view, in the analysis of biological samples for pharmacokinetic studies, the assay results with $\leq 10\%$ relative standard deviation and $\leq 10\%$ bias may be consid-

ered acceptable. Analysts should always attempt to improve the precision and accuracy of their assays, although a higher relative standard deviation and bias may be acceptable when the concentrations of the drug analyzed are extremely low.

4.7.5. Extraction recovery

An initial isolation of drug and metabolites in biological samples by means of extraction, filtration, protein precipitation or other procedures is usually performed before these samples are chromatographed to separate the drug, metabolites and the internal standard. Sometimes, a combination of multiple isolation procedures is necessary in order to allow adequate chromatographic separation. Many of these sample preparation procedures cause a loss of the drugs or metabolites due to incomplete extraction, adsorption, volume loss or co-precipitation. It is seldom that the drugs can be completely recovered from the biological samples, but the reduced recovery should be minimized by optimizing the conditions for the isolation procedures.

Like precision and accuarcy, recovery may be concentration-dependent and need to be evaluated over the range of the expected concentrations in the samples The extraction recovery can be estimated by comparing the slope of the calibration curve based on the calibration standards prepared according to the assay precedures, except that these standards do not contain internal standard during the sample preparation, with the slope of the calibration curve based on pure standard solutions of the drug. The same amount of internal standard can be added to the prepared calibration standards and drug standard solutions before chromatography to estimate the slopes of the calibration curves using peak-height (or -area) ratios. The ratio of these slopes is the assay recovery and sometimes is called absolute recovery. The recovery of the internal standard should be studied separately by comparing the peak heights or areas from the prepared samples containing internal standard and the stock solution of the internal standard. Recovery should also be reproducible. An assay method with low but reproducible recovery may be acceptable when the samples are analyzed with appropriate calibration standards However, good precision and accuracy cannot be obtained when the recovery of the analytes is not reproducible.

In some literature reports, the concentration of drug found in seeded control samples expressed as the percentage of the known or true drug concentration is called "recovery". By this definition, recovery is the same as accuracy and this is the reason that accuracy is reported as "recovery" by some analysts. As defined, the recovery should be close to 100%. However, recovery experiments are usually done when the assay method is being developed or validated The purpose is to obtain an overall high recovey of drugs, preferably $>75\%$, from the samples taken through the complete analytical procedures. Accuracy, on the other hand, is continuously monitored as the samples are analyzed.

4.8. Quality control and ruggedness of analytical methods

The effectiveness of the various applications of the analysis of the concentrations of drugs and toxic substances in biological samples in drug discovery, development and therapy, toxicology, and environmental research has made the importance of the accuracy of drug analysis self-evident. For routine analysis of high-volume samples, it is also important to be able to maintain accuracy and precision over a long period of time so that the assay results are reliable and not drifting. This can be achieved through the implementation of a quality control program to monitor the performance of the analytical procedures. A deficiency in documentation of quality control data in clinical pharmacology literature was recently identified and was attributed to the general attitude of investigators functioning as authors, referees and editors [176]. Many methods for the detection of drifting of assay results in quality control are currently in use and the principle and practice of quality control has been reviewed [1,8,9,228,229]. Among the most commonly used methods are the quality control chart methods. These control charts are variably called Levey–Jenning chart or Shewhart chart [182,183,230]. More recently, a modified time series modeling for quality control for clinical chemistry replacing a single control chart with a common cause chart and a special cause chart has been proposed [231].

For these procedures, seeded control or quality control samples containing known concentrations of the drugs are analyzed daily or with each run or each batch of the samples analyzed. The means and standard deviations of the quality control samples at each concentration level for each assay run are calculated. These values are plotted over time in the control charts. The mean value is represented as a horizontal line together with parallel lines of ± 2 standard deviations (warning limits) and ± 3 standard deviations (action limits) which approximate the 95 and 99% confidence limits, respectively. When the concentrations found in the quality control samples are scattered about the previously established means and within the warning limits, the assay procedures are under proper control. If one quality control result falls outside the action limits or two consecutive results fall outside the warning limits but within the action limits, the assay results are not rejected, but nevertheless the assay procedures are checked for instrumental or procedural errors, The assay method is no longer under statistical control when two successive quality control results fall outside the action limits and the assay results should be rejected and the assay procedures should be investigated for causes of the large deviations of the assay results [182].

The concentrations of many commonly monitored drugs in biological samples have been determined in different laboratories using a variety of analytical techniques. For the assay of these drugs, external quality control schemes are available. For example, external quality control schemes have been reported for the analysis of theophylline [232], tricylcic antidepressants [233] and antiepileptic drugs [234]. The external quality control procedures not only can detect drifting

or trend of analytical results, but also can assess both laboratory and analytical method biases.

Quality control is an *a posteriori* approach to detect trend and bias of analytical methods. It is also desirable to have an *a priori* approach to get an idea of the reliability of the analytical results that can be expected. This can be estimated by measuring the effect of small variations in the assay method on the assay sensitivity. The preferable property that an assay is not subject to small changes in the procedures is called ruggedness. A measure of ruggedness gives an indication of the day-to-day and inter-laboratory variations that can be expected. A factorial experiment has been proposed [235] to examine factors (*e.g.* HPLC columns, mobile phase pH, etc.) affecting the analytical sensitivity, and to evaluate the ruggedness of the assay method. This approach has also been used to optimize the analytical procedures and to identify critical factors that should be controlled more strictly to maintain the stability of the analytical methods.

4.9. Comparison of performance of analytical methods and laboratories

The need to compare analytical methods and laboratories arises when one wants to choose from among several available methods and laboratories to conduct sample analysis and to make conclusions based on the data generated by different assay methods or data from different laboratories using the same or different methods. For example, much lower assay results were obtained by an HPLC method compared with an microbial assay (slope of linear regression = 0.32) of the antibiotic itraconazole [236], total and free quinidine concentrations determined by FIA and HPLC were comparable, although FIA may have higher blank values than HPLC [237], and the potency of urokinase was essentially identical as determined by bioassay and by HPLC [238]. In addition, often the pharmacokinetic conclusions are drawn based on the plasma concentrations of drugs determined by different laboratories using different analytical methods. The latter includes many commonly monitored drugs such as the tricyclic antidepressants, digoxin, theophylline, anticonvulsants and others [10,14,239,240]. When an assay method is to be replaced by a new method because of the potential improvement in assay operational and performance characteristics (speed and ease of procedures, cost, precision and accuracy, etc.), one also compares analytical methods. Assay precision and accuracy are usually the most important criteria for comparison of the performance of different analytical methods and different analytical laboratories.

The simplest means of comparison of analytical methods or laboratories is to analyze standard or reference samples with known drug concentrations. The differences between the concentrations found and the true concentration are a measure of the accuracy, and the variations of the results of replicate assays are a measure of the precision of the methods or the laboratories Unfortunately, for these comparisons the standard or reference samples are often not available. It is,

therefore, most useful to analyze replicates of the same set of samples which contain a range but unknown concentrations of the drugs with different methods or in different laboratories. The results can be analyzed statistically. The most commonly used approach is the linear regression analysis of the one set of assay results *versus* the other. Ideally the linear regresion line should have a slope of unity and should pass through the origin. Both the slope and the intercept can be evaluated by confident limits estimation [185]. The correlation coefficient also has an ideal value of unity [241]. To detect interference, non-linearity and misuse of the imprecision component in method comparison, a maximum likelihood fitting of a functional relationship and weighted regression have been proposed as alternatives to the simple linear regression [242]. Other methods used are the paired *t*-test of the results from two methods or two laboratories for comparison of the means and the *F*-test for comparison of the precision [182,183].

4.10. Analytical data system and management

Chromatographic and other analytical procedures in many bioanalytical laboratories have been automated to different degrees, ranging from microprocessor-controlled instruments that perform a single specific function to integrated laboratory robotic systems that perform the entire sequence of the assay procedures. With the automation of analytical procedures, a large number of biological samples can be analyzed rapidly but the resulting analytical data and information can easily overwhelm the analysts. Fortunately, the advances in computerized data acquisition, analysis and management systems are matching the advances in automation of analytical instrumentation.

Acquisition of analytical data using chromatographic methods has evolved from mechanical and electronic integrators to computers with sophisticated algorithms to identify the beginning and the end of chromatographic peaks and to draw appropriate baselines [179]. These computerized data systems vary from the simple personal computers to very large laboratory information management systems (LIMS) [243,244]. In many advanced data systems, the analog signals from the analytical instruments are digitized and stored on tapes for long-term storage and retrieval. From theses digitized records, chromatograms can be regenerated for analysis and review. The data of calibration, control and unknown samples are compiled into a data base for electronic transfer of the data for analysis. The analytical data from quality control samples provide a convenient way of monitoring the performance of the analysis. It is important to validate and maintain the integrity of the analytical data systems.

4.11 Enantiomeric drugs

Drug enantiomers can exhibit different pharmacokinetic and pharmacodynamic properties. The pharmacokinetic differences between enantiomeric forms

are observed in total body clearance of acenocoumarol, verapamil, tocainide, warfarin, disopyramide and other drugs [245–253]. For verapamil, ther stereoselective first-pass metabolism of the active (−)-verapamil resulted in a two- to three-fold smaller plasma concentration of the (−)-enantiomer after an oral dose as compared to the same total verapamil concentration after an intravenous dose. This is the reason that a higher total verapamil concentration is needed after oral dosing compared to intravenous administration of racemic verapamil to elicit the same cardiac PR interval prolongation [254,255]. Stereoselective renal clearance of the enantiomers of chloroquine, salbutamol and ibuprofen was also reported [256–258]. These differences in clearance are further complicated by inter- and intra-subject variations for some drugs [247,259]. In addition, plasma protein binding of the drug enantiomers can also be different, notably for acidic drugs [260,261] and some basic drugs [246,262–264]. The therapeutic activity of racemic drugs may reside in one of the enantiomers, while the other enantiomer may have reduced, different or undesirable acitivy. Examples of drug enantiomers with different pharmacodynamic properties are indacrinone, ketamine, pentazocine, propranolol, sotalol, tetramisole, pheneturide and verapamil [265–274]. Achiral drugs, *e.g.* debrisoquine, may also form enatiomeric metabolites [275] The pharmacokinetic and pharmacodynamic differences between drug enantiomers have been reviewed [276–279].

Although most of the enantiomeric drugs are still administered as racemic mixtures, the dilemma is whether each of the enantiomers of the racemic drug in biological samples should be assayed for pharmacokinetic studies and for other applications or the current practice of monitoring total drug concentrations in the samples should be continued. With the currently available chiral separation techniques, it is rational to conduct studies to evaluate the potential pharmacokinetic and pharmacodynamic differences between drug enantiomers. The pharmacodynamic and pharmacokinetic evaluation can be done by evaluating each enantiomer separately in a cross-over study or, preferably for pharmacokinetic studies, by administering the racemic drug and using a chiral assay method to analyze the samples for each enantiomer. If there are known clinically significant differences between the enantiomers with regard to their pharmacokinetic or pharmacodynamic properties, then one is obliged to analyze the concentrations of the enantiomers in pharmacokinetic studies.

Enantiomers can be separated either by using a chiral environment or by using achiral conditions to separate the diastereomers after derivatization of the enantiomers with a pure chiral reagent [280–282]. Enantioselective chromatography and radioimmunoassay have been used to measure the concentrations of enantiomers in biological samples after administration of racemic drugs [283]. Gas and liquid chromatographic columns containing chiral stationary phases are effective means for the separation of optical isomers. Pirkle-type and cyclodextrin HPLC columns have been made commercially available for several years [283,284]. More recently, α_1-acid glycoprotein and bovine serum albumin col-

umns have been developed [285,286], and other chiral stationary phases for HPLC columns have also been reported [287–289]. The addition of chiral complexing agents or optically pure ion-pairing agents in the HPLC mobile phase [290] likely promotes *in situ* formation of transient diastereomers between the chiral reagents and the enantiomers to allow the separation of the enantiomers on achiral columns. Precolumn derivatization of enantiomers with chiral reagents [291–293] to form diastereomers, which are separated on achiral columns, is an effective and widely used method. However, precolumn derivatization requires the enantiomers to have reactive functional groups suitable for derivatization and optically pure chiral derivatization reagents. The large number of recent publications on chromatographic assay of enantiomers [290–300] attests to the high interest and activities in this area. HPLC and GC enantioseparation has been reviewed [280–283]. Enantioselective RIA uses antisera against pure enantiomers and optically pure labeled radioligands. Using this technique, measurement of enantiomers of phenobarbital [301–302], hexobarbital [303] and warfarin [304], after the racemic drugs were given to man and animals, have been reported.

4.12. Free versus total drug concentrations

Some drugs are highly bound to plasma proteins. The drug-binding proteins are mainly albumin for acidic drugs and α_1-acid glycoprotein for basic and some acidic drugs. The concentrations of these drug-binding proteins may change under different physiological and pathological conditions and therefore may affect the extent of binding of drugs. Protein binding of drugs may also be concentration-dependent. It is generally assumed that only the unbound or free drug is pharmacologically active [46,305–307]. Because of these reasons, determination of the concentration of free drug in plasma samples for therapeutic drug monitoring or pharmacokinetic evaluation has been proposed [308,309]. However, determination of free drug concentration can be justified or is useful probably only in limited cases. The reasons for the need to measure free drug concentration in plasma samples based on pharmacokinetic and pharmacological considerations have been reviewed [307]. It was proposed that determination of free drug concentration is warranted only for drugs showing extensive binding to protein ($\geq 80\%$), likely variable fraction unbound within the therapeutic concentration range under the physiological and pathological conditions, small volume of distribution (≤ 2 l/kg), good correlation between pharmacologic effect and free concentration, and narrow therapeutic index [307]. Some drugs that have been recommended or have showed valid utility of determination of free concentrations are valproic acid, phenytoin, carbamazepine, lidocaine and disopyramide.

Analytically, determination of free drug concentration in plasma or serum samples presents additional technical challenges as well as cost in that the free and bound drug must be separated and the analytical method for the assay of the total concentration may need refinement to allow for the measurement of the

lower concentration of the free drug compared to total drug concentration. The established methods for separation of free and protein-bound drugs in plasma are equilibrium dialysis, ultrafiltration and ultracentrifugation. Equilibrium dialysis has been the most often used method. However, it is time-consuming and cannot be used for drugs with high non-specific binding to the dialysis cells or membrane. The other two methods also have advantages and limitations. After separation, the concentrations of free drugs are analyzed by chromatographic or other suitable bioanalytical methods. Separation and determination of free drug concentrations have been reviewed [305–307,310–313].

It is also interesting to note that highly protein-bound drugs may show reduced recovery under certain analytical conditions. Using column-switching and on-line precolumns for trace enrichment of drugs in directly injected plasma samples for reversed-phase chromatographic separation, highly protein-bound drugs such as naproxen, amitriptyline and retinoids were found to have low recovery because of the loss of drugs in the enrichment step [314,315]. The recovery was found to be inversely proportional to the degree of drug–protein binding in the sample. Methods that decrease the degree of protein binding such as dilution with buffer, pH adjustment and protein precipitation increase the assay recovery. An increase in the residence time of drug on the precolumn by increasing the length and hydrophobicity of the precolumn also improve the assay recovery of highly protein-bound drugs in plasma.

5. ALTERNATIVE METHODS FOR ANALYSIS OF DRUGS AND OTHER TOXIC SUBSTANCES IN BIOLOGICAL SAMPLES

Although the chromatographic assay methods have been used extensively to analyze drugs and other substances in biological samples for pharmacokinetic studies and other applications, various techniques of immunoassays have also been utilized for these purposes. For some special applications such as therapeutic drug monitoring, immunoassays have become very popular and have replaced HPLC in the analysis of some drugs in biological samples because of the speed, automation and the simplicity in sample handling. RIA utilizes antibodies raised against drug hapten-protein conjugates and radiolabeled drugs. Under favorable conditions, RIA can measure drug concentrations in serum two to three orders of magnitude lower than the most chromatographic assays. For example, concentrations as low as 4 pg of fluphenazine [316] and 40 pg of sulforidazine [317] and mesoridazine [318] in 200-μl plasma samples were measured by RIA procedures. Pure radiolabeled ligands are required for all RIAs. In addition, some RIA methods have comparatively poor precision and are subject to nonspecific interference and varying cross-reaction with drug metabolites and other unrelated compounds. The unpredictable immune responses to the protein conjugates of drugs also make the standardization of RIA very difficult. The use of monoclonal antibodies can assure the availability of more reproducible binding

agents, but the binding specificity cannot be assured. Due to the variable cross-reactivity, the cyclosporin concentrations in blood determined by using different specific and non-specific polyclonal and monoclonal antibodies were highly variable [319], and the assay results of a RIA procedure using one clone of the specific monoclonal antibodies correlated better with the results from an HPLC assay than other clones [320]. Diphenhydramine, although structurally unrelated to theophylline, was reported to interfere with a RIA procedure for theophylline [321]. Other potential problems with the immunoassay of digoxin have been reviewed recently [322].

Because of the cross-reactivity observed with most antisera, the assay specificity of RIA procedures is always a concern. This has prompted many studies to compare RIA with other more specific assay methods. The plasma concentrations of fluphenazine and sulforidazine determined by the RIA procedure were consistent with the HPLC assay results [316,317], but the RIA-determined plasma concentrations of mesoridazine showed a high bias (about 10% by the slope of linear regression) compared to the HPLC assay results [318], and the concentrations of flupentixol determined by RIA also biased high (about 20% by the slope of linear regression) compared to the results from a GC procedure [323]. Comparable concentrations of leukotriene B_4 in human neutrophil samples over the range 0.1–100 ng/ml were obtained by RIA and GC–MS procedures [324], although at the low end of the concentration range, the RIA results tended to show a high bias compared to the GC–MS method.

In addition to radiolabeled ligands for RIA, many alternative labels for ligands have been developed for immunoassays. Among the alternatives, the enzyme labeling for EIA and the fluorescence labeling for FIA are more commonly used for analysis of drugs in biological samples. More recently, chemiluminescence and bioluminescence immunoassay procedures have also been developed [325–327]. Procedures for both the EIA and FIA have been developed for many drugs and, like RIAs, many commercial kits for analysis are available for various drugs [1]. Because these EIA and FIA assay procedures also depend on competitive binding of labeled and non-labeled ligands to antibodies, the assay specificity is not always assured. Comparative studies have indicated that the concentrations of valproic acid in serum measured by a FIA procedure were in good agreement with the concentrations determined by a GC method [328]. Plasma levels of amitriptyline, nortriptyline, imipramine and desipramine determined by an automated EIA (EMIT) procedure and by an HPLC method were in good agreement and showed similar precision and accuracy [329]. However, the plasma quinidine concentrations measured by an EMIT procedure were 1.2-fold higher than results from a specific HPLC method [330].

Chromatographic separation has been used to fractionate angiotensin II peptides in human plasma followed by RIA to allow sensitive and specific measurement of the peptides [331]. Alternatively, procedures using tandem immunoaffinity and conventional HPLC have been developed for analysis of theophylline and polypeptides and other drugs [116,120,332,333].

Other frequently used alternative assay methods for drugs in biological samples are RRA and MS RRAs are similar to RIA except that the naturally occurring receptors instead of antibodies are used for binding of the ligands. RRA procedures have often been used for analysis of anticholinergics, β-blockers and benzodiazepines [334–337]. Using glucose-6-phosphate dehydrogenase-labeled phencyclidine and isolated acetylcholine receptors from *Torpedo nobiliana*, an enzyme-amplified receptor assay for drug analysis was reported [338]. MS coupled with GC or HPLC has increasingly been used for assays of drugs in biological samples because of its inherent specificity and sensitivity. MS is the method of choice for identification of drug metabolites in drug metabolism and excretion studies [339]. Drugs labeled with stable isotopes have been used in pharmacokinetic, bioavailability, bioequivalence and drug interaction studies which offer the advantages of avoiding intra-subject variations and time effect as encountered in the conventional cross-over studies. The assay procedures using MS are excellent for determination of drug concentrations in samples from these studies with stable isotope-labeled drugs [340–344]

6. TRENDS IN BIOANALYSIS

Although bioanalysis is an exciting and fast evolving subject, its future development cannot be predicted. However, it is apparent that some technologies are actively being pursued, setting a trend that may have a major impact on bioanalysis in the near future. In recent years, mathematical, statistical and other methods have been increasingly employed to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information from the analytical data. This formal logic approach to analytical chemistry is termed chemometrics [182]. Currently chemometrics is a fluid subject, but it is having impact on all aspects of analytical chemistry [345,346]. Statistical methods for analytical chemistry and other aspects of chemometrics have been reviewed [345–347]. Applying chemometrics to characterize the assay reliability of analytical methods has been proposed [348]. For bioanalysis, assay method development and validation, quality control, laboratory automation and data management are already utilizing principles of chemometrics. The future should see the growth and development of chemometrics into a mature subject [346] having greater impact on all areas of analytical chemistry including analysis of drugs and toxic substances in biological samples.

Biological sensors have been rapidly developed during recent years, and more practical applications in the analysis of drugs and other substances in biological samples may be found. Current status and future trends in biosensors and their applications to drug analysis have been reviewed [349–353]. For practical application, a naproxen ion-selective electrode was constructed and utilized in a dissolution study of a naproxen tablet [354]. A biosensor based on acetylcholine receptor protein isolated from a *Torpedo* electric organ was built [355]. When

tested with acetylcholine, other neurotransmitters and esterase inhibitors, *d*-tubercurare, amantadine, and α-neurotoxin, the response of the biosensor closely resembled that of the nicotinic acetylcholine receptor [355]. Measurement of electroactive antibiotics *in vivo* in the bloodstream of rats using a catheter electrode was recently reported. This *in vivo* voltammetric method was utilized to evaluate the pharmacokinetics of defsulodin, chloramphenicol, metronidazole and nimorazole in rats [356]. The concentrations of these antibiotics determined by the *in vivo* voltammetry correlated very well ($r > 0.99$) with the results from HPLC assays of simultaneously collected blood samples [356].

New technology for the separation, measurement and data handling in the analysis of drugs in biological samples has been developed rapidly. Although sample preparation technology has not kept pace, advances have been made in the direct introduction of biological fluids for analysis of drugs [123,127–129], the automation of on-line column-switching for extraction and enrichment of analytes [123-125] and in the use of laboratory robot for sample preparation [357,358]. An apparent trend is the combination of the sample preparation, chromatographic separation and data handling technologies to fully automate bioanalysis to enhance quality and productivity in the laboratory [359]. As potent new drugs are being discovered and developed, urgent health and environmental issues are to be evaluated, and sophisticated management of drug therapy and patient care emerges, bioanalysts will be required to rapidly provide precise and accurate measurement of lower and lower levels of drugs and other toxic substances in biological samples to meet the demand of the applications. The analysis of drugs, their metabolites, endogenous compounds and other toxic substances in biological samples will continue to have a major contribution and impact on pharmacokinetics, clinical pharmacology, health and environmental research.

7 SUMMARY

The importance of the role of analysis of drugs and other toxic substances in biological samples (bioanalysis) in medicine, toxicology, pharmacology, forensic science, environmental research and other biomedical disciplines is self-evident. Among these disciplines, bioanalysis plays a special pivotal role in pharmacokinetics. The pharmacokinetic parameters, such as half-life, volume of distribution, clearance and bioavailability, of drugs and other compounds are derived from the concentrations of these analytes assayed in the biological samples collected at specified time points. The capability of analysts to develop sensitive and specific analytical methods for the assay of low concentrations of drugs and other toxic compounds in small amounts of biological samples has contributed significantly to the theoretical advances in pharmacokinetics and its applications in clinical pharmacology and the management of drug therapy in patients. The increased demands for pharmacokinetic applications in turn have stimulated the innovation and improvement in bioanalytical technologies.

The reliability of the pharmacokinetic conclusions depends on the accuracy and precision of the analytical methods employed to assay the biological samples. Factors that affect the integrity of the bioanalytical data should therefore be controlled in analysis of biological samples for pharmacokinetics studies. The biological samples for drug concentration determination should be collected as specified in the study protocol with respect to the time and site of sampling. These samples should be processed to avoid extraneous interactions between the analytes and sampling devices or additives resulting in the redistribution of the analytes between components of the biological samples, such as displacement of drug binding and changes in the distribution of the analytes between plasma and red blood cells. The stability of the drugs and other analytes in the samples should also be evaluated to establish the conditions suitable for the transportation and storage of the samples to avoid chemical, photochemical and enzymatic degradation of the analytes.

Various technologies have been utilized to assay biological samples for pharmacokinetic studies. The most frequently used are chromatography (high-performance liquid chromatography, gas chromatography and thin-layer chromatography), immunoassays and mass spectrometry. Except for some immunoassays, the biological samples are usually prepared by liquid–liquid or solid-phase extraction taking advantage of the pH ionization and partition characteristics of the analytes for preliminary separation of the analytes from other components in the sample matrices. Such extraction procedures should be optimized to obtain high and, most importantly, reproducible recovery. Approaches to simplify the sample preparation procedures have been actively pursued and some advances such as immunoaffinity purification prior to chromatographic separation or other means of quantification and direct introduction of the biological samples for chromatography using column-switching techniques and special stationary phases have been made.

Validation of bioanalytical methods is an important task to assure the reliability of the assay data. In addition, validation of analytical methods has also been required by various regulatory agencies. Such validation generally requires the demonstration of the specificity, sensitivity, calibration linearity, extraction recovery, precision and accuracy of the assay methods However, despite the importance of assay method validation, many of these terms have been defined differently and the criteria for validation of bioanalytical methods are often dependent on the intended application of the assay. For pharmacokinetic evaluations, it is most important that the analytical methods should be specific with no interferences from endogenous and exogenous substances; that the methods should have a low limit of quantification capable of accurate measurement of the compounds over a sufficient period of time following drug dosing or exposure necessary to characterize the pharmacokinetic parameters; and that the accuracy and precision of the assay are demonstrated by appropriate calibration standards and seeded control samples.

Analysis of drugs and other toxic substances in biological samples is a challenging task because the concentration of the analytes in the complex matrices are often very low. The interest in free drug concentrations in plasma further requires that the assay method is sensitive to measure even lower concentrations of the free drugs and metabolites. The renewed interest in stereoselectivity of drug disposition has also added the demands for measurement of drug enantiomers. New bioanalytical technologies in chromatography, immunoassays, radioreceptor assays, mass spectrometry and biological sensors are emerging. These, together with the development in automation in bioanalytical laboratories in sample preparation, analysis and data management, provide analysts with an ample armamentarium to meet the present analytical requirements for pharmacokinetic studies. These changes also present an opportunity for innovation and improvement of the bioanalytical technologies to meet the ever demanding requirements of pharmacokinetics and other biomedical sciences that rely on accurate measurement of lower and lower levels of drugs and toxic substances in biological samples.

8 ACKNOWLEDGEMENT

The authors thank J. E. Katz for her assistance in editing the manuscript.

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