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REVIEW

ANALYTICAL STRATEGIES FOR THERAPEUTIC MONITORING OF DRUGS IN BIOLOGICAL FLUIDS

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1. INTRODUCTION

Although the chemical structure of a drug largely dictates the most suitable assay procedure for its determination [1,2], the magnitude and frequency of the doses administered and the pharmacokinetics of the compound govern the ultimate sensitivity and specificity required of an assay for its quantitation in biological fluids. These criteria have to be elucidated relatively early in the life of a compound being developed for clinical evaluation. Since the ultimate clinical efficacy of a new drug substance depends largely on the absolute bioavailability of its dosage forms [3], and the pharmacokinetics of its disposition in man (the ultimate target) [4], the compromise between assay sensitivity and specificity can be justified depending on the stage of development of the drug [5].

An effective new drug development program requires the successful coordination of multi-disciplinary activities once the pharmacology and acute toxicology of the compound has been determined and a decision is made to initiate chronic toxicity studies prior to clinical testing. At this stage the generation of relevant biopharmaceutical and pharmacokinetic information relies heavily on the expeditious development of a suitable chemical assay for the drug candidate. The compound is usually extracted from the biological matrix (blood, plasma, serum) followed by sample "clean-up" and possible derivatization to enhance sensitivity of detection and/or selectivity for analysis [2].

Therapeutic drug monitoring can involve quantitation in the microgram or ppm, nanogram or ppb* or picogram or ppt concentration range.

The degree of "clean-up" required is dependent on the analytical method used [gas—liquid chromatography (GLC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC)] and on the tolerance of the specific type of detection system to contamination. The options available for processing a biological specimen must be tailored not only to the method itself, but also to the sensitivity and specificity required of it [6]. Factors responsible for compound losses during sample preparation (adsorption, stability) are critical at low concentrations and may adversely affect the reliability of an assay. Consequently, maximizing the overall recovery of the analyte is essential not only for sensitivity but also for good precision and accuracy.

The choice of the method selected is governed either by the intrinsic analytical properties of the molecule or its amenability to chemical derivatization to

*Throughout this article, the American billion (10^9) and trillion (10^{12}) are meant.

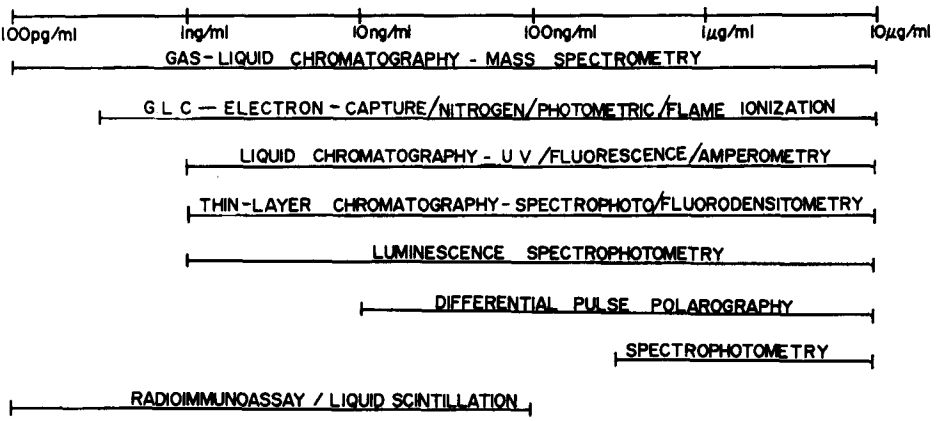


Fig. 1. Practical range of usefulness of analytical techniques.

render it compatible to quantitation by a specific method [7,8]. The type of assay selected will also govern the amount of sample preparation and "clean-up" required, and the biological specimen best suited for analysis, i.e., whole blood, plasma, or urine [6].

The armentarium of sensitive and specific methods currently available to the analyst is quite diverse and covers a wide linear dynamic range for quantitation (Fig. 1). These include chromatographic techniques [2] with a variety of selective detectors to ensure specificity, e.g., GLC with ionization detection such as electron-capture (ECD), nitrogen—phosphorus-specific detection (NPD), chemical ionization mass spectrometric detection (CI-MS), HPLC with UV, fluorescence and electrochemical detectors (oxidative and reductive polarographic), high-performance TLC (HPTLC) with "in situ" spectrophoto/fluorodensitometry or non-chromatographic techniques such as spectrophotometry (UV—VIS), luminescence methods (fluorescence and phosphorescence), differential pulse polarography (DPP) and radioimmunoassay (RIA), all of which are capable of quantitation over a wide linear dynamic concentration range.

2. CHEMICAL STRUCTURE AND ANALYTICAL UTILITY

Drug molecules have certain intrinsic properties which allow for their detection by physico-chemical means. The majority of the compounds are aromatic (benzenoid) or heterocyclic in nature and usually possess functional groups such as $-NO_2$, $-NH_2$, $-OH$, $>C=O$, $-CHO$, halogens (F, Cl, Br, I), $-COOH$, $-CH_2OH$, $-OCH_3$, $=N-CH_3$, $-CHOH$ which influence the physico-chemical properties of a molecule and are also amenable to micro derivatization reactions that are suitable for analysis by any one or several of the techniques outlined in Fig. 1.

The following general properties which influence the aromaticity ("π"-electron-rich systems) of the molecule are conducive to increasing the UV absorption and the luminescence emission properties of drug compounds:

(a) Fusion of the benzene ring to other benzene rings, e.g., naphthalene, anthracene, phenanthrene, etc., or to heterocyclic rings as in the quinolines, isoquinolines, indoles and carbazoles, phenothiazenes, benzodiazepines.

(b) Electron-donating functional groups on the aromatic nucleus such as amino, dimethylamino, alkyl side chains, alkoxy, phenoxy, tend to increase UV absorption and the intrinsic luminescence of the molecule by increasing its "π"-electron density.

(c) Certain electron-withdrawing functional groups such as the nitro, halogens, carbonyl, azomethine, nitrile, and carboxyl, tend to decrease UV absorption and the intrinsic luminescence of the molecule due to delocalization of the "π" electrons in the benzenoid or heterocyclic nucleus. However, they are highly sensitive to either GLC—ECD, GLC—CI-MS or DPP analysis.

Chemical manipulation of a drug via derivatization is useful in enhancing both the sensitivity and the specificity of the determination step [7,8], and has certain inherent advantages even if the intrinsic sensitivity is adequate and/or blood concentration is not a limiting factor. The sample volume extracted can be reduced from milliliter to microliter amounts, and/or the aliquots of the final residue analyzed can be reduced by sample dilution, significantly improving chromatographic analysis by minimizing endogenous interferences, resulting in more accurate, reproducible and reliable quantitation. All of the above factors should be optimized in the overall development of a reliable and validated method for eventual clinical evaluation.

2.1. Spectrophotometric characteristics

The UV absorption and luminescence behavior of a compound in a given solvent can be used to advantage in determining its ionization characteristics,

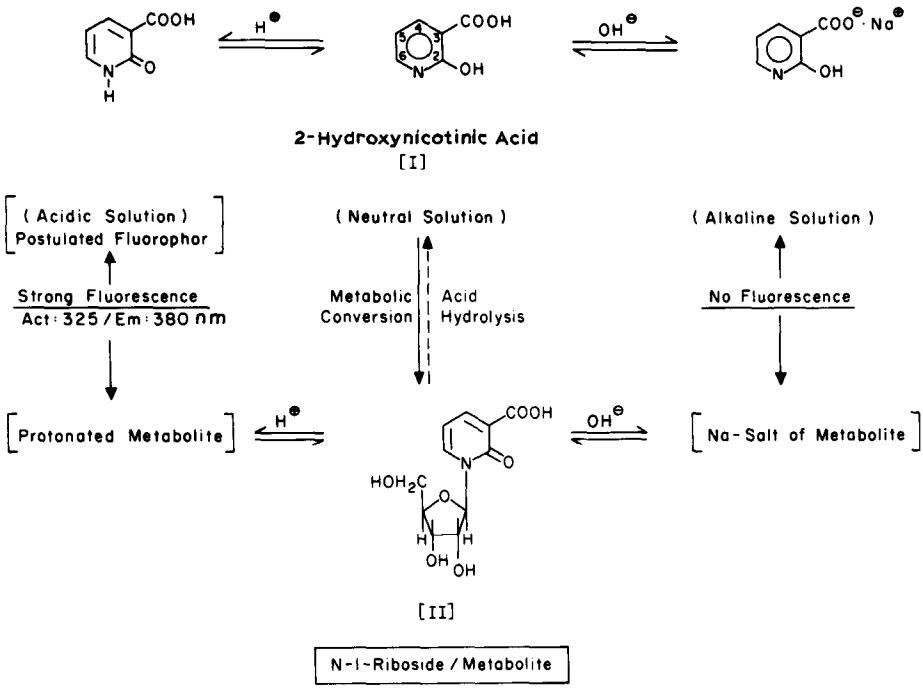


Fig. 2. Influence of pH on the luminescence characteristics of 2-hydroxynicotinic acid (a hypolipemic agent) and its major metabolite. Act = activation; Em = emission.

as indicated by shifts in (a) the wavelength ($\lambda_{max.}$) of maximum absorption and changes in (b) its molar absorptivity.

The absorption spectrum of a compound is markedly influenced by the nature of the solvent (polar or non-polar) and the pH of the medium used. Polar solvents can alter the spectrum due to hydrogen bonding or even chemical reaction with the compound. The pH of the medium can significantly change the spectral characteristics due to ionization of the molecule to form cationic or anionic species in solution resulting in spectral shifts to either longer wavelengths (bathochromic or red shift), or to shorter wavelengths (hypsochromic or blue shift). Changes in spectral position ($\lambda_{max.}$) can also be accompanied by either an increase (hyperchromic effect) or a decrease (hypochromic effect) in absorptivity. These properties are used to determine the ionization characteristics (pK_a) and the isosbestic pH of the drug and are essential in determining the pH for optimal extraction into an organic solvent. The influence of pH on the luminescence of 2-hydroxynicotinic acid (a hypolipemic agent) is shown in Fig. 2. Spectral absorbance and luminescence properties of a compound may be optimized for sensitive and/or selective detection of a drug by HPLC analysis.

3. EXTRACTION OF THE ANALYTE FROM A BIOLOGICAL MATRIX

Whole blood, plasma or serum is the biological specimen usually analyzed for therapeutic drug monitoring. The sample volume required depends on the absolute concentration present (governed by the clinical dosage regimen and the pharmacokinetics of the drug), and the absolute sensitivity of the analytical method to be used. Chemical assays (GLC, HPLC, GLC—MS) usually require 1—2 ml of biological sample (requiring venipuncture) whereas biological assays (RIA, immunological and microbiological assays) can be performed with sample volumes of the order of 10—50 μl readily obtained by capillary blood sampling techniques preferred in pediatric and geriatric clinical practice. Clinically valid correlations of blood concentration data can be obtained from either capillary blood sampling or venipuncture procedures. The former procedure can circumvent unnecessary trauma to the patient [9].

Extraction of the analyte usually involves either organic solvent extraction at a pH at which the analyte is $>99\%$ unionized (basic drugs at pH >6, and acid drugs at pH <5.5), or by protein precipitation using either inorganic salts such as tungstate, phosphate or chloride or organic solvents such as acetonitrile, acetone, ethanol or trichloroacetic acid (TCA). The analyte in the solvent extract or protein-free filtrate is determined by a suitable method usually following "clean-up" and preconcentration depending on the concentrations present or instrument sensitivity desired. The use of "solid extraction" methods can also accomplish selective extraction, "clean-up" and preconcentration with a minimum of sample manipulation expediting sample throughput. The use of adsorbent cartridges containing a plug of reversed-phase (C_{18}) HPLC packing material has found commercial application as solid extractants. An automated centrifugal type batchwise 12 sample extractor/concentrator (DuPont Auto Prep II®) was used to analyze several anticonvulsants from serum using 100 μl to 2 ml of sample (depending on concentration) with recovery $>99\%$. The extracts were analyzed by reversed-phase HPLC and cor-

related well against GLC and enzyme-multiplied immunoassay technique (EMIT) analysis. A variety of adsorbent cartridges (e.g., ion-exchange resins, reversed-phase C_{18} packing), makes it a viable procedure [10].

Solid-extraction columns containing C_{18} packings (e.g., Bond Elut® from Analytichem International, U.S.A.) contained in polyethylene hypodermic syringes with luerlock fittings (1 ml capacity) are convenient for batchwise manual use. Small sample volumes (<500 μl) of blood, plasma or serum are passed through the column, which retains the analyte, which is later stripped off with a suitable eluent, and readily reconstituted in 25—50 μl of mobile phase for reversed-phase HPLC analysis. Benzodiazepines have been successfully analyzed with $>90\%$ recovery making the technique suitable for use where small sample volumes are required [11,12].

The next step in automating sample handling procedures would have to be the use of robots; e.g., Zymate® (Zymark Corp.) interphasing sample preparation with analytical quantitation, data reduction and documentation [13].

4. METABOLIC PROFILE

Metabolic studies on the "in vitro" biotransformation of the compound using microsomal (9000 *g*) enzyme preparations and/or "in vivo" studies using the radioisotopically labelled (^{14}C, ^{3}H) compound should be underway in parallel with method development, to elucidate the in vivo metabolic profile of a drug in blood and urine so that assay development can also incorporate the quantitation of the major metabolites present in blood or urine using more sensitive and specific chemical methods.

The extent of metabolism, especially "first pass" effects as it relates to overall bioavailability and clinical effectiveness, should be defined early. Depending on the functional groups present in the compound, sites of metabolic attack such as $-OCH_3$, $-N-CH_3$, $-OH$ (phenolic or alkoxy), $-COOH$, $-NH_2$ (aromatic or aliphatic), $-COCH_3$, a compound can undergo extensive biotransformation by $-O$ or $-N-$dealkylation, to form metabolites which can undergo either O- or N-glucuronidation, or hippurate formation to form water-soluble conjugates which are eliminated in the urine. Consequently the concentration of the parent drug may be limiting in both blood and urine, whereas that of the conjugated metabolites may be quite high. Thus, the analysis of urine may be more practical from the point of view of sensitivity and if one metabolite predominates "specificity" can be achieved by diluting out the minor metabolites. The quantitation of the major metabolite (hydroxyethylflurazepam) in urine derived from the parent drug (flurazepam) could substantiate the bioavailability of that drug [14]. If the parent drug is not extensively biotransformed and/or distributed in body tissues, then its quantitation in whole blood, plasma or serum should be feasible, especially after chronic dosage when "steady state" concentrations have been established.

It should be noted that selective extraction of a given drug to the total exclusion of its unconjugated metabolites be they polar or non-polar, basic or acidic, is usually not feasible. Some cross-contamination is inevitable, and becomes even more problematic, especially when the ratio of parent drug to metabolites is 1:1 or greater. Thus absolute specificity is difficult to achieve by selective

solvent extraction alone [15] except in cases where markedly different molecular structures are involved. Absolute specificity for a known drug and its metabolites usually also requires a chromatographic separation step to ensure it.

Determination of the "total" radioactivity in plasma or urine vs. solvent extractable radioactivity will indicate the extent to which polar non-extractable metabolites are present. Chromatographic analysis (TLC or HPLC) of the solvent-extractable fraction using either a radiochromatogram scanner or a radiometric detector with HPLC analysis will indicate the relative amounts of the parent drug and any metabolites present; a reliable index of the specificity of the extraction procedure (pH and solvent used).

Characterization of the chemical structure of the predominant (key) phase I metabolites using GLC—MS, NMR and the synthesis of authentic reference compounds will enable their quantitation in biological fluids using sensitive and specific chemical methods [16].

Chromatographic analysis is necessary to ensure the specificity of analysis for the parent drug and/or any major metabolites present. Any "first pass" biotransformation will be reflected in the ratio of parent drug to major metabolites; indeed the metabolite may be the only measurable component present; e.g., rapid hydrolysis of an ester to an acid, and can influence the choice of the biological sample to be used. Radioisotopic data would also indicate the feasibility of developing a chemical assay for the compound in terms of the ultimate sensitivity and/or specificity required of it.

Therefore, knowledge of the metabolite profile of a drug in blood and urine of both the "free" or directly extractable and the "bound" or conjugated metabolite fractions and their relative distribution in these media is invaluable in "tailoring" the specificity requirements of an assay to the task at hand.

5. DEVELOPMENT OF A SUITABLE ANALYTICAL METHOD

Based on the intrinsic physico-chemical properties of the drug candidate, its pK_a value, solubility in organic solvents, partition characteristics and most probable pathways of biotransformation (based on previous experience with analogous compounds) a decision has to be made as to which analytical method (TLC, GLC, HPLC) would yield a suitably sensitive and adequately specific assay for parent drug and its probable major metabolites [1,2]. The overall recovery of the assay for varying concentrations of added authentic analyte taken through the entire procedure should be at least 75—80% with precision and reproducibility of replicate determinations within the run (intra-assay variability) and between determinations on separate days (inter-assay variability) being within ±10% (S.D.) or ±2 (S.D.) about the mean. These statistical parameters should be evaluated against an absolute standard such as ^{14}C- or ^{3}H-labelled drug which would normally be available for metabolic studies. Criteria for the development of analytical methods for drugs and their metabolites have been previously discussed [1—3,6].

The primary focus of therapeutic drug monitoring is on the parent drug and its pharmacokinetic profile in man. The concentration of the parent drug in blood may be limiting irrespective of the administered dose since biopharmaceutical factors such as dissolution rate, pK_a, permeability across the lipoidal

mucosa, crystalline state (polymorphism) and pharmacokinetic factors such as extensive "first pass' metabolism in the gut wall and/or in the liver, a high volume of distribution and/or rapid elimination in the urine (conjugates) and in the feces via biliary excretion may result in low parent drug concentration in the systemic circulation. Conversely, it may result in the presence of a major active metabolite of the drug wherein the parent compound functions as a pro-drug (e.g., Clorazepate — nordiazepam, acetylsalicylic acid — salicylic acid, hetacillin — ampicillin). One has to be alert for such circumstances and it is advantageous to have authentic standards available for some of the more probable metabolites that may be identified.

Quantitation of the parent drug and/or its major metabolites usually requires a chromatographic separation step (TLC, GLC or HPLC) to ensure specificity.

The quantitation of metabolites is relevant only if they are either pharmacologically active or if their accumulation leads to toxic side effects or even a reduction in the activity of the parent drug due to competitive displacement from the receptor sites. Inactive and/or innocuous metabolites do not warrant quantitation and their inclusion may even confuse the issue, especially with a non-specific assay. The poor correlation obtained between the microbiological assay vs the fluorometric assay for ampicillin [17], was due to the fact that the former responded only to ampicillin (the active compound) hence specific, while the latter was non-specific and involved chemical oxidation to a fluorphor common to the parent drug and its inactive penicilloic acid (Fig. 3). Thus plasma concentrations determined by the microbial assay were specific for the active component but yielded lower concentration (μg/ml) values than the fluorometric assay which represented the sum of two components.

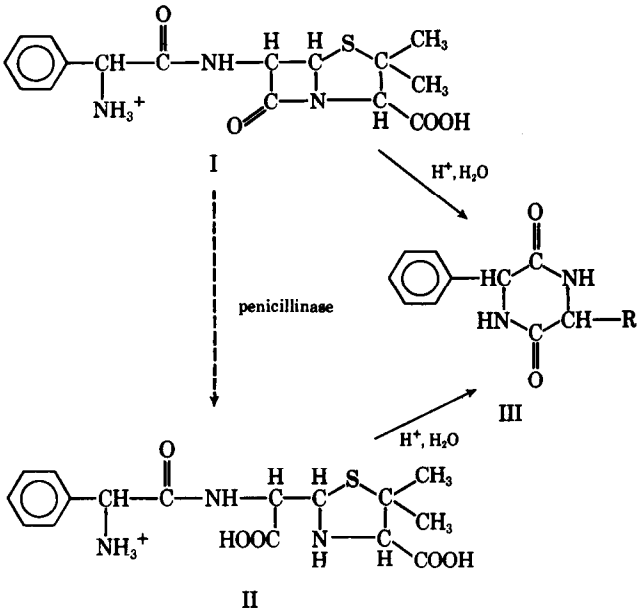


Fig. 3. Proposed pathway for formation of a 3,6-disubstituted diketopiperazine [III] from ampicillin [I] and/or α-aminobenzylpenicilloic acid [II] [17].

Thus, the correct interpretation of blood antibiotic concentrations with antimicrobial activity can be influenced significantly by the specificity of the assay used. HPLC analysis was most advantageous in the specific determination of several antibiotics in the presence of their inactive metabolites by the direct analysis of human serum and urine using ion-pair [18], and reversed-phase [19] chromatography using either UV detection at 254 nm or fluorometric detection following post-column derivatization with fluorescamine or *o*-phthalaldehyde [20]. An excellent correlation for tetracycline determined in human serum using a microbiological assay vs. reversed-phase HPLC with fluorometric detection (intrinsic fluorescence of tetracycline) was also reported [21], showing the validity of the less sensitive yet specific microbiological assay for antibiotics.

6. PHARMACOKINETIC FACTORS WHICH INFLUENCE SAMPLE PREPARATION

6.1. Biotransformation

Chromatographic analysis is necessary to ensure the specificity of analysis for the parent drug and/or any major metabolites present. Any "first pass' biotransformation will be reflected in the ratio of parent drug to major metabolites; indeed the metabolite may be the only measurable component present and may dictate the choice of the biological sample to be used, e.g., rapid hydrolysis of an ester to an acid. Radioisotopic data would also indicate the feasibility of developing a chemical assay for the compound in terms of the ultimate sensitivity and/or specificity required of it.

6.2. Elimination

The rate and extent of elimination of a drug and/or its metabolites in urine would dictate the utility of analyzing this medium. Drugs that are extensively metabolized by phase I reactions are eliminated in urine following phase II reactions as the glucuronide—sulfate—hippurate conjugates [16]. Their concentrations are usually sufficiently high to warrant their analysis in urine as in bioavailability—bioequivalence studies for dosage-form evaluation.

7. SAMPLE PROCESSING VS. ANALYTICAL DETERMINATE STEP

7.1. Analysis of blood/plasma and tissue

The degree of sample preparation and "clean-up" required is usually a function of the analytical method to be used and the tolerance of the specific detection system to contamination. The options available for processing whole blood/plasma or tissue homogenate or proteolytic digest are outlined in Fig. 4. The biological specimen undergoes a protein precipitation step, followed by pH adjustment and selective extraction into a suitable solvent, which can then be processed in one of several ways depending on the analytical method to be used [6].

Radioimmunoassay (RIA) can be performed either directly in the biological

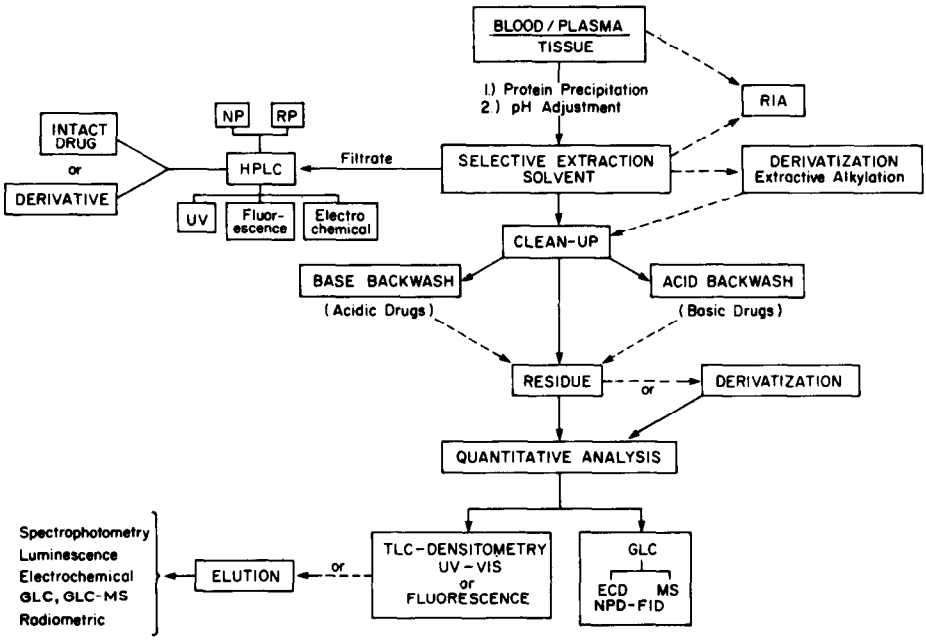


Fig. 4. Flow diagram of the analytical options available for sample processing of blood/plasma or tissue homogenates. NP = Normal-phase; RP = reversed-phase; FID = flame ionization detection; NPD = nitrogen—phosphorus detection.

sample (plasma or serum) or in the residue of the solvent extract which can also be used for HPLC analysis.

HPLC analysis has several advantages that can be collectively optimized for sensitive and specific analysis. Two modes of operation, normal-phase (adsorption) and reversed-phase (partition) are the most widely used for drug analysis, although cation/anion-exchange chromatography is also used for highly polar zwitterionic drug molecules such as the β-lactam antibiotics and quaternary (NH_3^+) compounds [2]. The sample residue is reconstituted in 50—100 μl of the mobile phase to be used and passed through a 40-μm Millipore filter to remove colloidal lipids and lipoproteins which could precipitate out if a reversed-phase system is used. Drug molecules can be analyzed either as the intact moiety or as a suitable derivative using either UV, fluorescence or electrochemical detection.

GLC analysis may require more extensive sample preparation depending on the drug to be analyzed. The drug in the solvent extract can be derivatized directly by extractive alkylation (N-1-desalkyl-1,4-benzodiazepin-2-ones) followed by a "clean-up" step, or the intact moiety can be further purified from endogenous impurities by utilizing a backwash step into a base (for acidic drugs) or an acid (for basic drugs), and re-extracted from the aqueous phase by appropriate pH adjustment into a solvent, the residue of which may be derivatized prior to GLC analysis (silylation, esterification, alkylation) using highly selective detection methods such as ECD, NPD or CI-MS.

TLC analysis may be performed directly on the residue of the initial solvent extract, unless pre-chromatographic clean-up is required due to a derivatization

step. TLC separation can be used, per se, as an effective clean-up step since the extract applied to the chromatoplate can be processed by multiple development, first in a non-polar lipophilic solvent system to move the endogenous contaminants from the origin to the solvent front followed by a second development, in a more polar solvent to resolve the compounds of interest from each other. The compounds can either be quantitated "in situ" by spectrophotometry (UV—VIS) or fluorodensitometry or be eluted from the silica gel and determined separately by an appropriate physico-chemical method (spectrophotometry, luminescence, electrochemical, radiometric).

7.2. Analysis in urine and feces

A flow diagram for the extraction of urine and feces is outlined, in Fig. 5. The analysis of these two media requires an aliquot of a representative sample, i.e., an aliquot of urine from a total voidance volume collected over a known excretion period (e.g., 24 or 48 h), and for feces an aliquot of a homogenate of a total voidance collected similarly. The sample is filtered to remove particulates and analyzed for the "free" or directly extractable and "bound" or conjugated fractions of drug and metabolites present.

Basic extracts of urine or feces are not as heavily contaminated with endogenous compounds as are acidic extracts which have extensive amounts of phenolic and indolic acids, and require additional "clean-up", e.g., column chromatography. The conjugated or "bound" fraction has to be hydrolyzed either with acid to cleave hippurates and other amino acid conjugates or incubated with β-glucuronidase—sulfatase at 37°C for 2—12 h in a Dubnoff incubation shaker to cleave glucuronide—sulfate conjugates. The aglycones are extracted after appropriate pH adjustment, followed by "clean-up" of the

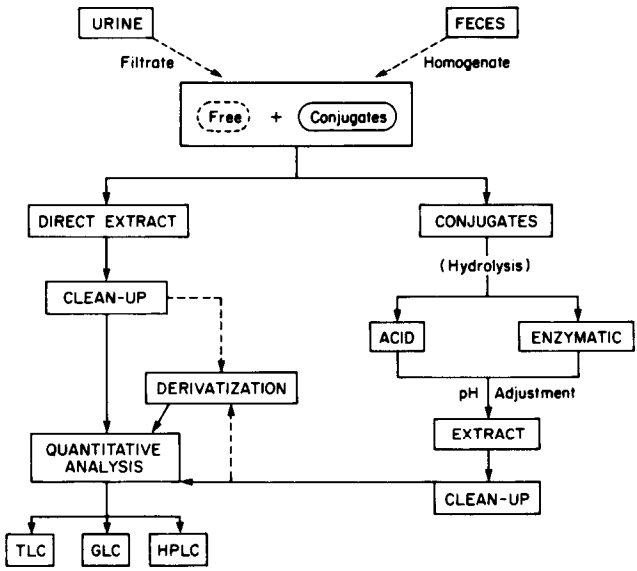


Fig. 5. Sample preparation of urine and feces for the extraction of drugs and their metabolites.

extract either by chromatography (column or TLC) or liquid—liquid partition (acid—base).

Depending on the determinate step, the residue of the final extract may have to be derivatized (silylation of hydroxyl groups, esterification of carboxylic acids) for GLC analysis, or analyzed per se by either TLC—densitometry or HPLC (usually reversed-phase) using UV, fluorescence or electrochemical detection. HPLC is generally the method of choice since non-volatile or thermally labile components can usually be analyzed without derivatization and in samples where concentration is not limiting. Resolution of endogenous impurities not removed by previous clean-up may be a limiting factor. Analytical problems encountered in sample processing for quantitative analysis of drugs in biological media was recently reviewed [22].

7.3. Sample collection devices

The type of collection device into which the biological sample is drawn should be evaluated as part of method development for a specific drug and its metabolites. The selection of glass or plastic tubes for blood collection and subsequent separation of plasma or serum and its effect on the drug in an "in vivo" sample with respect to adsorption losses, contamination by plasticizers leaching from rubber stoppers (e.g., B-D vacutainer tubes), displacement of protein bound drug by plasticizers and their effects on the plasma to haematocrit ratios warrant investigation. Drug concentration data can be adversely biased by these phenomena, hence also their pharmacokinetic implications [23].

8. NON-CHROMATOGRAPHIC (DIRECT) ANALYTICAL TECHNIQUES

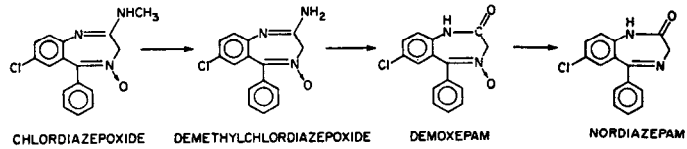
8.1. Absorptiometric and luminescence methods

Spectrophotometric (UV—VIS) and luminescence emission (fluorescence and phosphorescence) analysis [24], have been the classical methods used extensively in drug analysis. They possess adequate sensitivity but lack high specificity since spectral characteristics per se cannot usually differentiate the parent drug from any metabolites present unless used in conjunction with either differential/selective extraction techniques using liquid—liquid [15] or solid extraction [10,11], and/or a chromatographic separation step e.g., GLC—photometric detection, TLC or HPLC [2,3].

8.2. Differential pulse polarography

Electrochemical methods have better specificity by virtue of the functional group(s) in the molecule involved [25].

Polarographic methods have been used to advantage for the determination of the excretion of urinary metabolites of the 1,4-benzodiazepines, due mainly to the facile reduction of the azomethine ($>C_5=N_4-$) group common to these compounds [26] (Fig. 6). Hydroxyethylflurazepam and hydroxymethylmidazolam are the major urinary metabolites of flurazepam and midazolam,



Functional Group Reduced	CHLORDIAZEPOXIDE	DEMETHYLCHLORDIAZEPOXIDE	DEMOXEPAM	NORDIAZEPAM
$-N_4 \rightarrow O$	-0.275	-0.275	-0.315	-
$>C_4=N_5-$	-0.600	-0.590	-0.640	-0.645
$>C_1=N_2-$	-1.135	-1.020	-	-

Fig. 6. Differential pulse polarographic peak potentials (V vs. standard calomel electrode) of the reducible functional groups in chlordiazepoxide and its main metabolites in plasma determined in 0.05 *M* sulfuric acid [26].

respectively, and account for 30—40% of a given dose in a 24-h excretion period. The respective aglycones can be readily extracted following β-glucuronidase incubation and analyzed by DPP with a sensitivity of 0.50 μg/ml. Minor metabolites can be effectively diluted out yielding a specific assay for the major metabolite. Nitro group-containing 1,4-benzodiazepines e.g., nitrazepam, clonazepam, flunitrazepam can likewise be analyzed selectively using the NO_2 group reduction potential without interference from their respective 7-amino and 7-acetamido metabolites which do not respond at this potential.

8.3. Immunological assays

8.3.1. Radioimmunoassays

RIA is especially useful in monitoring drugs in pediatric anticonvulsant therapy where small volumes are necessary. Radioimmunoassays have recently been developed which are both very sensitive and specific for the parent drug in the presence of its major metabolites and/or other drugs administered concomitantly.

Ingenuity is required in the chemical synthesis of the hapten to ensure specificity to the major portion of the parent molecule in order that the antibody produced can distinguish it in the presence of its major metabolites. RIA development is a relatively complicated, time-consuming, and somewhat uncertain process. A successful RIA development program requires: (a) the chemical synthesis of a hapten of the drug which can be covalently coupled to a protein, usually bovine serum albumin (BSA) to produce the immunogen, (b) inoculation of a suitable animal species (rabbit or goat) with the immunogen (usually intradermally) over a period of at least 4—6 weeks to produce the anti-serum, (c) chemical synthesis of high-specific-activity drug; usually ^{3}H or ^{125}I labelled for use as the displacing agent to be bound to the anti-serum, (d) the entire system has to be tested for specificity, sensitivity, and freedom from cross-reactivity due to metabolites or other drugs that may be used concomittantly.

RIAs for benzodiazepines [27] are particularly useful in monitoring pediatric patients on anticonvulsant therapy which usually involves multiple drug regimens and the need for small (μl) sampling techniques. A novel method for the analysis of diazepam in either 10-μl samples of blood or plasma or 100-μl samples of saliva following oral administration of a single 5-mg dose of Valium® was described [28]. The blood samples obtained by heel or finger stick were absorbed onto filter paper at the clinic and mailed to the assay laboratory without any specific storage precautions. The drugs were subsequently leached with 1 ml of phosphate-buffered saline and analyzed directly by RIA. The technique is readily amenable for toxicological overdosage/drug compliance screening and for other clinical monitoring needs.

8.3.2. Radioreceptor assays

This relatively new biological assay technique resulted from the pharmacologists search for specific binding sites or receptors in the brain for benzodiazepines which might differentiate the anxiolytic from sedative properties of these compounds, and has been reviewed [29]. Radioreceptor assays for specific benzodiazepines have also been reported [30], and are being used to quantitate the total "active" fraction of parent drug and pharmacologically active metabolites in pharmacodynamic studies of anxiety vs. sedation. Depending on the class of compounds under investigation, meaningful correlations can be derived between plasma concentrations and the therapeutic effect elicited. These assays are finding expanded use in therapeutic drug monitoring.

8.3.3. Non-radioactive immunoassays

Alternative types of labels have been investigated in an attempt to retain the advantages of specificity and sensitivity of immunoassay without using radiolabels [31]. These include enzymes, fluorescent groups, stable free radicals, metals, erythrocytes and bacteriophages, all of which can be attached either to the hapten or to the antibody. A potential advantage of many (though not all) of the alternative labels is their amenability to homogenous assays, in which it is not necessary to separate antibody-bound from free hapten. This opportunity does not exist for radioimmunoassay since binding of antibody to an isotopically labelled antigen does not affect the radioactivity of the isotope. Of these, the enzyme immunoassays (EIA) and fluoroimmunoassays (FIA) are the most widely used in therapeutic drug monitoring. Enzyme immunoassays are classified into the heterogenous enzyme-linked immunosorbent assay (ELISA) and homogenous (EMIT) types and are differentiated as follows.

8.3.3.1. Enzyme immunoassays

8.3.3.1.1. Heterogenous enzyme immunoassay. In this type of assay the antigen to be measured is permitted to react with an excess of labelled antibody. After incubation excess solid-phase antigen is added to the mixture and binds any antigenfree labelled antibody present. Following separation of the solid phase, enzyme activity is measured in either the supernatant or the solid phase. The amount of enzyme activity present in the supernatant is directly related to the amount of antigen originally present in the sample (ELISA).

8.3.3.1.2. Homogenous enzyme immunoassay. In this type of assay (EMIT)

the omission of a phase-separation step is a major advantage in clinical use where speed is often essential. The principles of the EMIT® technique (developed by Syva Corp., Palo Alto, CA, U.S.A.) depend upon the fact that binding of antibody to an enzyme-labelled drug inhibits enzyme activity either by sterically preventing access of the substrate to the catalytic site or by inducing conformational changes in the enzyme which prevents catalysis. Free drug competes with enzyme-labelled drug for antibody with the result that enzyme activity is uninhibited and proportional to the amount of free drug originally present. The sensitivity of the method is limited by the antibody—antigen binding characteristics and the presence of background signals. It is, however, an effective method of determining the concentration of drugs in human serum with good precision and accuracy.

8.3.3.1.3. Characteristics of enzyme immunoassays. The performance of an enzyme immunoassay can be evaluated in terms of sensitivity, precision, accuracy, cross-reactivity and practicability like that of radioimmunoassay. The use of an enzyme label may introduce further complications, however; serum and plasma are more likely to affect the enzyme reaction but this can often be minimized by diluting the sample whenever possible, by measuring enzyme activity on the washed insoluble (bound) phase and by careful choice of enzyme. Heterogeneous enzyme immunoassays are of comparable sensitivity to the corresponding radioimmunoassay and are sufficiently sensitive to permit the determination of many clinically important drugs in biological fluids. Results obtained using enzyme immunoassay usually correlate well with those obtained using other immunoassay or reference techniques and both intra- and inter-assay coefficients of variation are generally within acceptable limits (i.e. ±10%). Homogeneous assays are generally much less sensitive than heterogeneous assays but, as already observed, have sufficient sensitivity to enable them to be used clinically with the added advantages of simplicity and rapidity.

8.3.3.2. Fluoroimmunoassays

Various types of fluoroimmunoassay have been developed, some of which require no separation step. The merits, in addition to its amenability to homogeneous assay, of fluorometric analysis are the stability and freedom from hazard of the fluorescent labelled materials, the moderate cost and wide availability of the equipment required and the high potential sensitivity [32].

The fluorescent label may function in a manner analogous to a radiolabel, i.e. the activity of the fluorophor is unaffected by its attachment to the antibody; therefore, a separation step is needed. Of more interest are assays in which the properties of the label are altered by binding to antibody: these require no separation step and may be both rapid and easily automated.

8.3.3.2.1. Polarization fluoroimmunoassay. This technique is based on the fact that when plane polarized light falls on an antigen labeled with a fluorophor, molecules with their long axes parallel to the plane of light are preferentially excited. If they also rotate between excitation and light emission the resultant fluorescence is non-polarized. The degree of depolarization depends on the rate of rotation and this, in turn, is inversely proportional to molecular size. The binding of a low-molecular-weight labelled antigen to anti-

body produces a large complex moiety which, unlike the native antigen, is only capable of rotating slowly so that much of the emitted fluorescence is still polarized. In an immunoassay unlabelled antigen present in the reaction mixture competes with the fluorescent-labelled antigen for antibody binding sites so that the extent of the depolarization of the emitted fluorescence is proportional to the concentration of antigen originally present in the solution. Commercially available assays in kits are marketed by Miles Labs. of Elkart Indiana as the Ames TDA® System which like the EMIT kits marketed by Syva Corp. are accurate, precise and easy to operate using the appropriate instrumentation e.g., Fluorostat® and Optimate® commercial systems.

8.3.3.3. Luminescent immunoassays

Luminescent (chemiluminescence and bioluminescence) immunoassays have attracted attention due to their potentially high sensitivity. In one variant a covalent complex of a chemiluminescent compound (a luminol derivative), a protein and a steroid hapten, was used as a labelled antigen analogous to the radio-label in competitive radioimmunoassay. The chemiluminescent label was bound by hapten-specific antibody and displaced by unlabelled steroid. Antibody-bound steroid was separated from the free steroid using a second antibody. Chemiluminescence was generated by oxidizing the label with hydrogen peroxide and copper acetate at high pH and the emitted light measured in a liquid scintillation counter. The assay was sensitive to 0.5 ng testosterone.

8.3.4. Advantages of the immunoassays

The advantages of immunoassay over other techniques for determining drug concentrations in biological fluids are that (i) they are usually very sensitive permitting determinations to be made on very small volumes of sample; (ii) they can generally be carried out on urine, plasma and saliva without prior extraction and purification; (iii) they are chemically gentle and can, therefore, be used to determine unstable or labile substances; (iv) they are potentially universally applicable to all classes of compound; (v) they are often quick and technically easy to perform, once the reagents have been prepared, and lend themselves readily to automation; (vi) they can be developed to produce assays that are remarkably specific or non-specific according to the preference of the investigator and the purpose for which the assay is designed. Virtually absolute specificity can be achieved, if desired, by combining solvent extraction or chromatographic separation with an immunoassay.

9. CHROMATOGRAPHIC TECHNIQUES

9.1. Gas—liquid chromatography

Although analysis of the intact molecular moiety (underivatized) is preferred to ensure specificity, derivatization is often necessary for valid analytical reasons. Simple derivatization reactions such as extractive alkylation and/or silylation can be used *where needed*, to yield very sensitive, specific and readily automatable methods. The idiosyncrasies of specific detection methods such as ECD and NPD have to be considered during sample preparation so as not to introduce contaminants into the extract which could be detrimental.

NPD, for example, is susceptible to severe interference by residues of silylating reagents and from phosphate plasticizers contained in plastic syringes and blood collection tubes which leach into the biological sample and are co-extracted. Thus, the selection of the proper type of syringe and collection tubes become a necessary part of the assay development program and should be evaluated with forethought so as not to jeopardize the clinical studies for which the assay is intended.

Microderivatization of a drug at low concentrations can be problematic in obtaining reproducible yields and in minimizing adsorption losses. Prostaglandin drugs, e.g., the antiulcer agent Trimoprostil®, administered in μg/kg doses results in plasma concentrations in the picogram range. GLC—negative-ion chemical ionization (NICI)—MS analysis of the drug as its trimethylsilyl, pentafluorobenzyl ester enabled its sensitive and specific quantitation with a sensitivity limit of 100 pg/ml of plasma [33]. Endogenous prostaglandins have likewise been determined using both capillary GLC—ECD and capillary GLC—MS analysis [34].

Where high sensitivity and resolution are required, GLC analysis using either ECD or NPD in conjunction with fused-silica capillary columns is the "state of the art" in GLC analysis, and with GLC—MS analysis. Fully automated GLC analysis from sample injection to final data reduction is now the generally accepted mode of analysis. Capillary fused-silica columns used in conjunction with NPD have found extensive application in monitoring tricyclic antidepressants (TCDs) with high sensitivity and specificity. Since numerous metabolites are commonly found at steady state especially with multiple drug therapy, the high resolution of the capillary column is essential. The small sample volume usually injected (1 μl or less) is a trade off to prevent column overload. The NPD system is stable even when the electrometer is operated at maximum sensitivity [35].

GLC—ECD analysis has been extensively used in the determination of the 1,4-benzodiazepines (Fig. 7) (which are widely used in clinical practice as anti-anxiolytics, muscle relaxants, hypnotics, and anticonvulsant agents) [36], due to the presence of an electronegative group in the 7-position of the molecule (usually a halogen or nitro group) (Fig. 8). A halogen in the 2′-position of the 5-phenyl ring, and a carbonyl group in position 2 of the 1,4-benzodiazepine ring also contribute to ECD response. Their extensive biotransformation in man results in the presence of one or more pharmacologically active metabolites which have to be resolved from the parent drug for accurate quantitation in biological fluids (Fig. 9).

Since the therapeutic doses for these compounds are usually low (generally less than 1 mg/kg in adults for single oral doses), analytical methods for their quantitation in biological media have to be both very sensitive and specific. GLC—ECD methods for the determination of drugs and metabolites were reviewed [37].

The properties of a drug molecule that render it amenable to GLC—ECD analysis can be readily adapted to the development of highly sensitive and specific GLC—CI-MS assays operated in either the positive ion (PI) or negative ion (NI) modes of analysis. The benzodiazepines lend themselves excellently to GLC—CI-MS analysis [38].

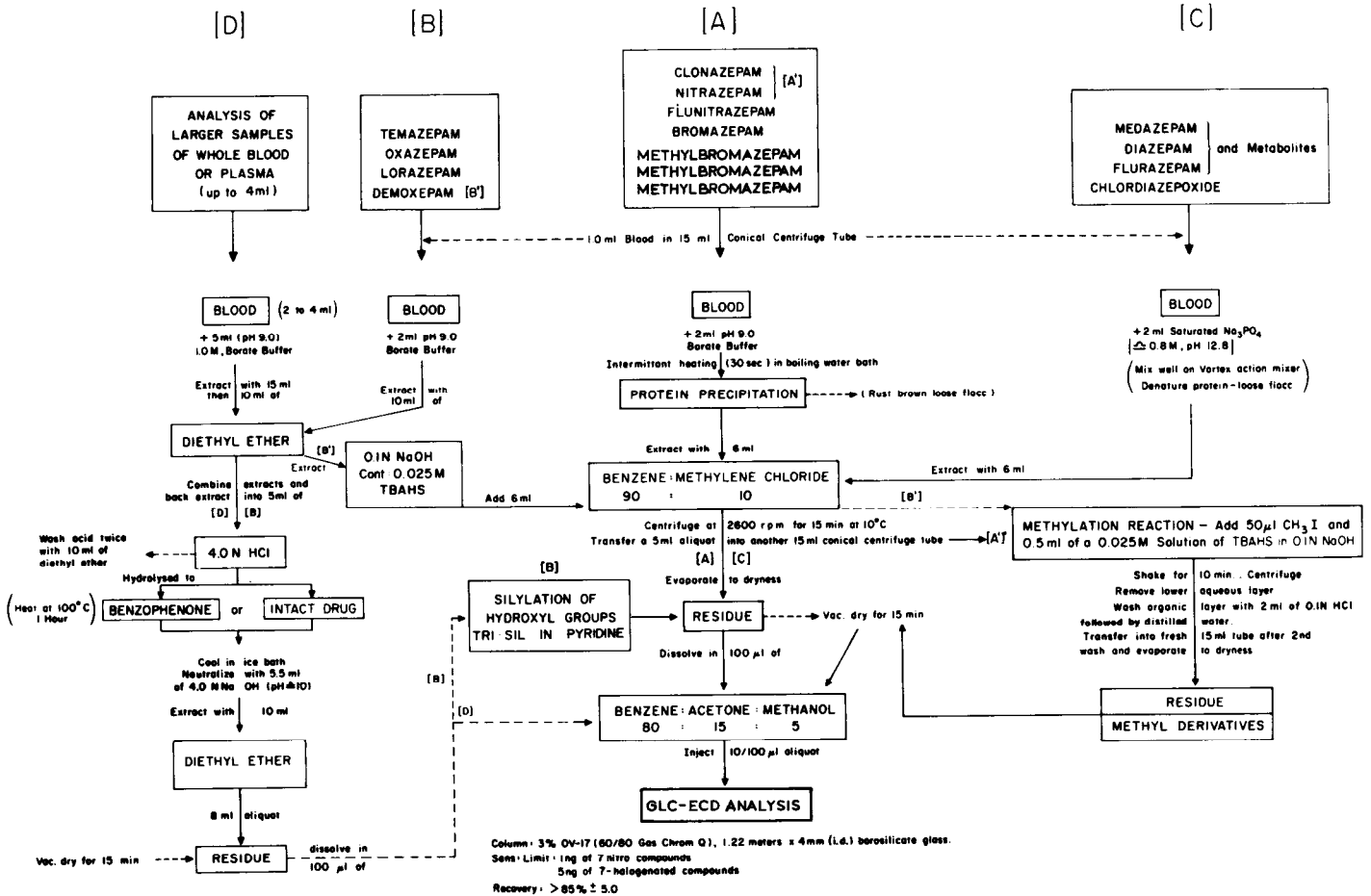


Fig. 7. Flow diagram of the extraction procedures used for the analysis of various 1,4-benzodiazepines and 1,4-benzodiazepin-2-ones in whole blood. THBAS = tetrabutylammonium hydrogen sulfate.

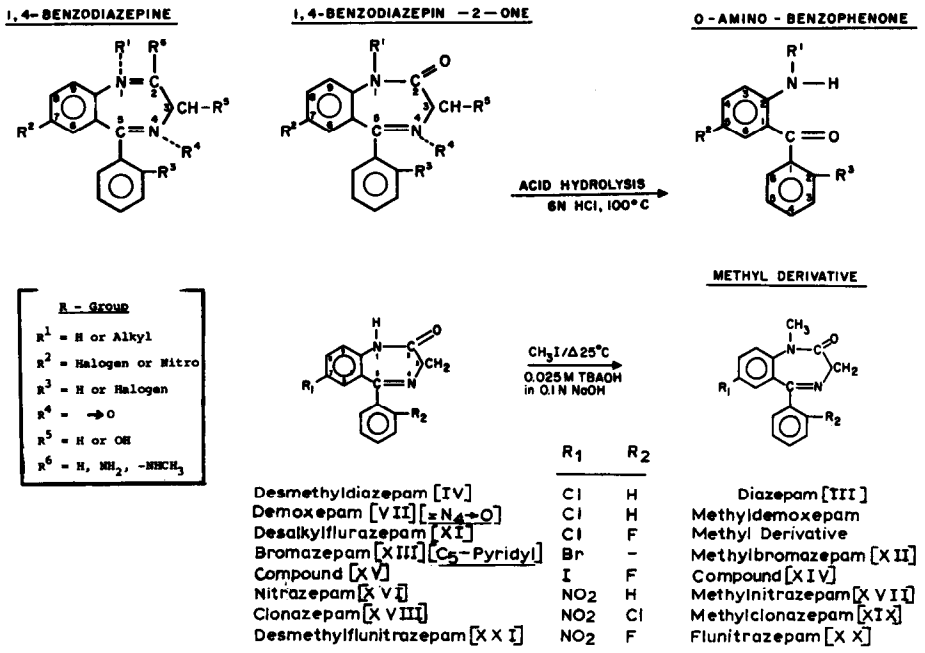


Fig. 8. Chemical reactions of 1,4-benzodiazepines and benzodiazepin-2-ones to yield derivatives suitable for GLC—ECD analysis. TBAOH = tetrabutylammonium hydroxide.

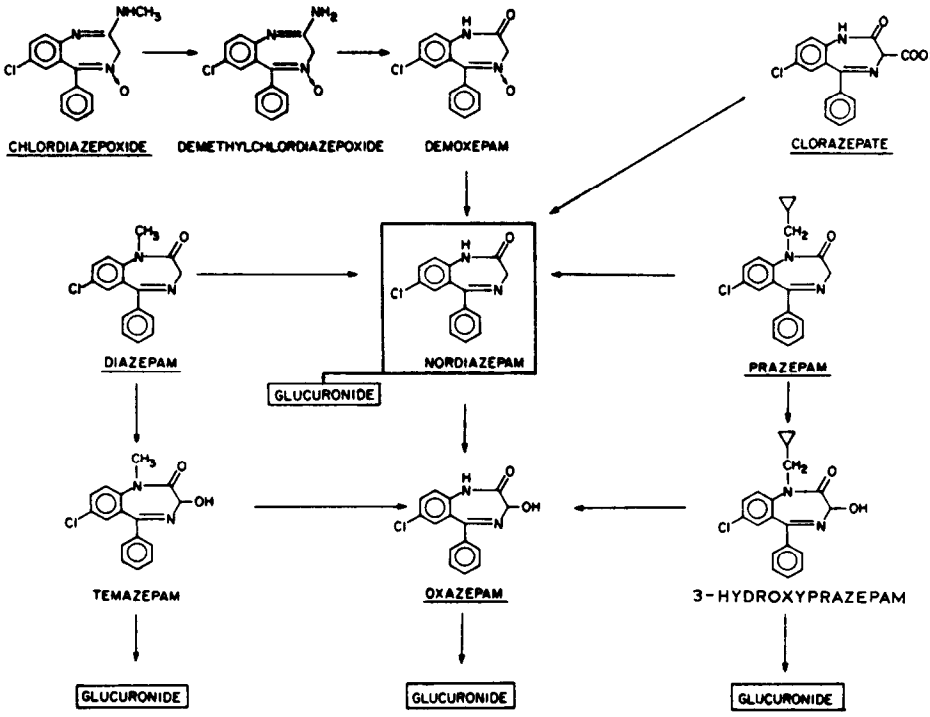


Fig. 9. Biotransformation of benzodiazepines to metabolites common to several drugs.

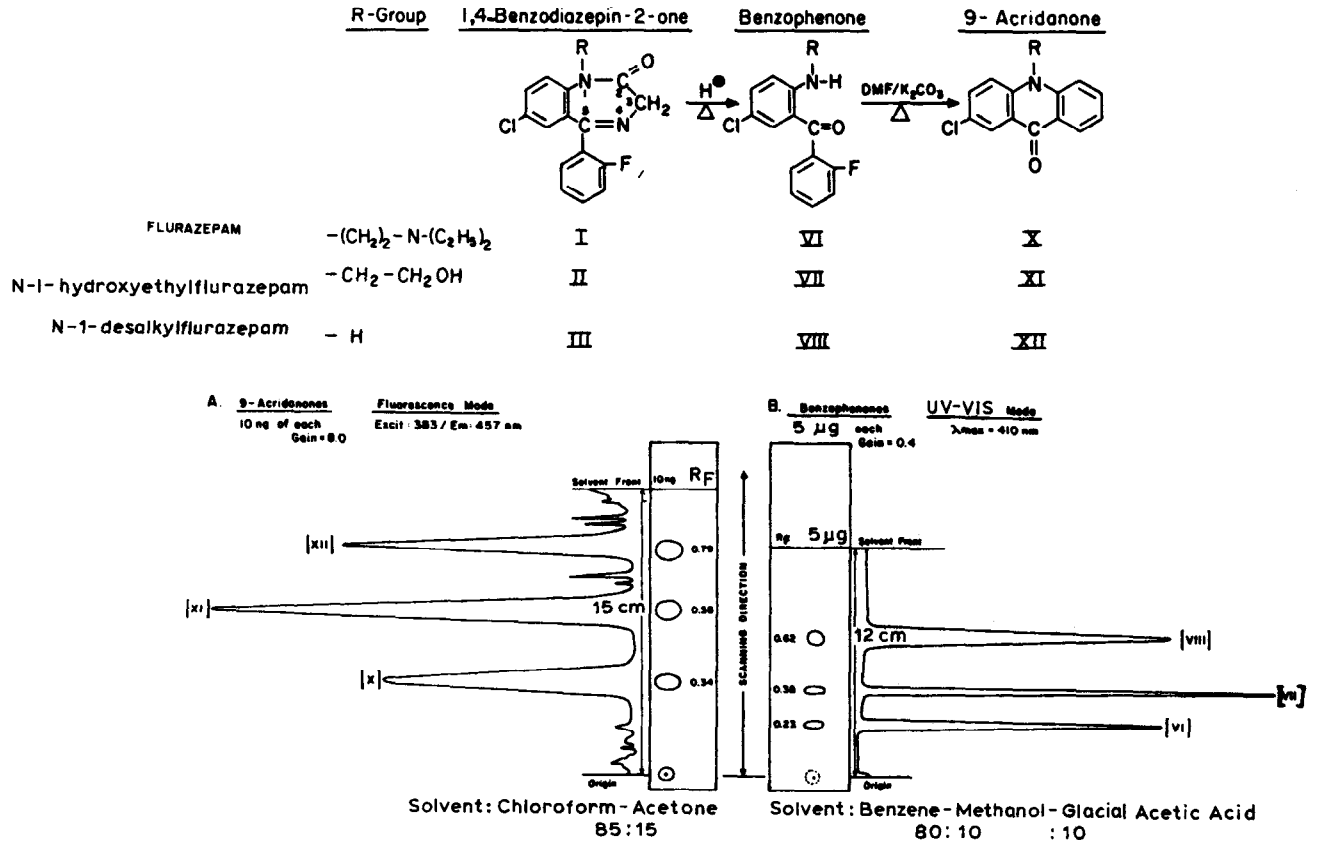


Fig. 10. TLC—densitometric analysis of flurazepam and its major metabolites in plasma as either the benzophenone (UV—VIS mode) or 9-acridanone (fluorescence mode) derivatives. Excit = Excitation; Em = emission. DMF = dimethylformamide.

9.2. *Gas chromatography—chemical ionization mass spectrometry*

GLC—CI-MS analysis has rapidly established itself as the definitive method for quantitation of drugs since greater sensitivity and specificity can be realized due to the milder reaction conditions used in the ionization source. The high abundance of either positive $[MH]^+$ or negative $[M-H]^-$ molecular ions generated yield a stronger signal (hence, greater sensitivity) and the ions formed are characteristic of the parent molecule, which coupled with selected ion monitoring (SIM) imparts greater specificity of analysis [39].

GLC—NICI-MS analysis of the 1,4-benzodiazepin-2-ones has inherently high sensitivity associated with negative-ion formation by electron capture in the CI source which can be 100—1000 times greater than that obtainable by positive-ion chemical ionization (PICI-MS) methods. This was demonstrated for the 7-nitro anticonvulsant, clonazepam which was analyzed by both GLC—PICI-MS [40], and GLC—NICI-MS [41]. The $[M-H]^-$ ion monitored at *m/z* 314 using the ^{15}N, ^{18}O, stable isotope analogue as the internal standard (*m/z* 321), yielded a sensitivity limit of 100 pg/ml in the NICI mode compared to that of 1000 pg/ml in the PICI mode monitoring the $[MH]^+$ ion at *m/z* 316.

The success of GLC—NICI-MS is also attributable to the development of quantitative micro chemical derivatization reactions which can convert compounds containing either an aromatic —OH or aliphatic —COOH, $-NH_2$ group to electron-capturing "electrophores" using either pentafluorobenzaldehyde, pentafluorobenzyl bromide or benzoyl chloride which can be quantitated with picogram sensitivity [42].

9.3. *Thin-layer chromatography*

TLC analysis enables rapid development of chromatographic parameters for eventual use in HPLC analysis. Preliminary separation of drug and metabolites using a radiolabelled compound enables qualitative identification via radiochromatographic scanning or autoradiography to isolate metabolites from either an "in vitro" 9000 *g* microsomal incubation or from "in vivo" biotransformation studies [16]. The separated compounds can be eluted from the silica gel and analyzed by a variety of selective techniques, e.g., spectrophoto/fluorometry, polarography, GLC, GLC—MS, NMR, not only for quantitation but also for structure elucidation purposes.

Quantitative analysis by "in situ" spectrophoto/fluorometry has been extensively utilized, especially since the advent of HPTLC using small sample aliquots 1 μl or less applied to the chromatoplate, rapid development and densitometric analysis [43]. The sensitivity and specificity of the technique is especially useful in the fluorescence mode and was used in the analysis of flurazepam and its major metabolites in plasma as their highly fluorescent 9-acridanone derivatives (Fig. 10). These were extracted, separated by TLC and quantitated by "in situ" spectrofluorodensitometry with picogram sensitivity, and applied to the determination of plasma concentrations of flurazepam and its major metabolites; hydroxyethyl and N-desalkylflurazepam following a single 30-mg oral dose of Dalmane® [I] · 2HCl [44].

Some of the advantages of TLC analysis include the ability of analyzing the

entire sample by either one- or two-dimensional solvent ascending preparative-scale chromatography, rapid development of the separation (HPTLC) and relatively low cost of the separations per se. This is unfortunately offset by the high cost of the spectrodensitometer required for quantitation.

9.4. High-performance liquid chromatography

HPLC analysis has several advantages that can be collectively optimized for sensitive and specific detection. Drug molecules can be analyzed either as the intact moiety or as a suitable derivative using either UV, fluorescence or electrochemical detection.

HPLC is uniquely suited to the analysis of thermally unstable compounds (e.g., the benzodiazepines chlordiazepoxide and its metabolites), and amphoteric "zwitterionic" compounds (antibiotics) which are difficult to extract, at best. Plasma and serum can be analyzed directly following protein precipitation with acetonitrile, injecting an aliquot of the protein-free filtrate (after partitioning with *n*-hexane as a clean-up step to remove colloidal lipids) [45]. Urine is filtered to remove salts and colloidal materials, and an aliquot diluted in the mobile phase and analyzed directly using reversed-phase HPLC.

Amoxicillin (a β-lactam antibiotic, pK_a = 2.4, 9.6, structurally related to ampicillin), and its benzyl-penicilloic acid, were analyzed directly in urine, by HPLC using fluorometric detection following post-column derivatization with fluorescamine [46].

Analysis in the fluorescence mode (excitation 385 nm, emission 490 nm) circumvented the need for extensive clean-up due to the selectivity of the detection system for the compounds of interest with minimal interference from endogenous materials.

High sensitivity and specificity can be achieved by tandem monitoring of the column effluent using either UV—fluorescence or UV—electrochemical detection. The use of an electrochemical detector for the determination of benzodiazepines in the reduction mode using a dropping mercury electrode (DME) [47] using the functional group specificity of the $\rangle C_5 = N_4-$ azomethine group attests to the utility of this technique (Fig. 11).

Electrochemical detectors have also found extensive application in HPLC analysis in the oxidative mode using a glassy carbon electrode especially in the analysis of catecholamines with excellent sensitivity in the picogram concentration range [48]. The oxidative detector is very cheap compared to UV, fluorescence or polarographic detectors, and its sensitivity has advanced the field of catecholamine biochemistry significantly. Whereas GLC—ECD or GLC—MS analysis requires derivatization of the phenolic and amine groups using silylating and perfluoro acylating reagents, HPLC analysis can achieve the same sensitivity of 5—10 pg/ml using the electrochemical detector in the phenolic group specific oxidative mode [49]. A variety of cell geometries used in solid state carbon electrodes (tubular, thin-layer and rotating disc) and DME types have been reviewed [50].

HPLC analysis has made very significant inroads into therapeutic drug monitoring which was once the monopoly of GLC, TLC or direct spectrophotometric methods. The popularity of the technique is largely due to its versatility in

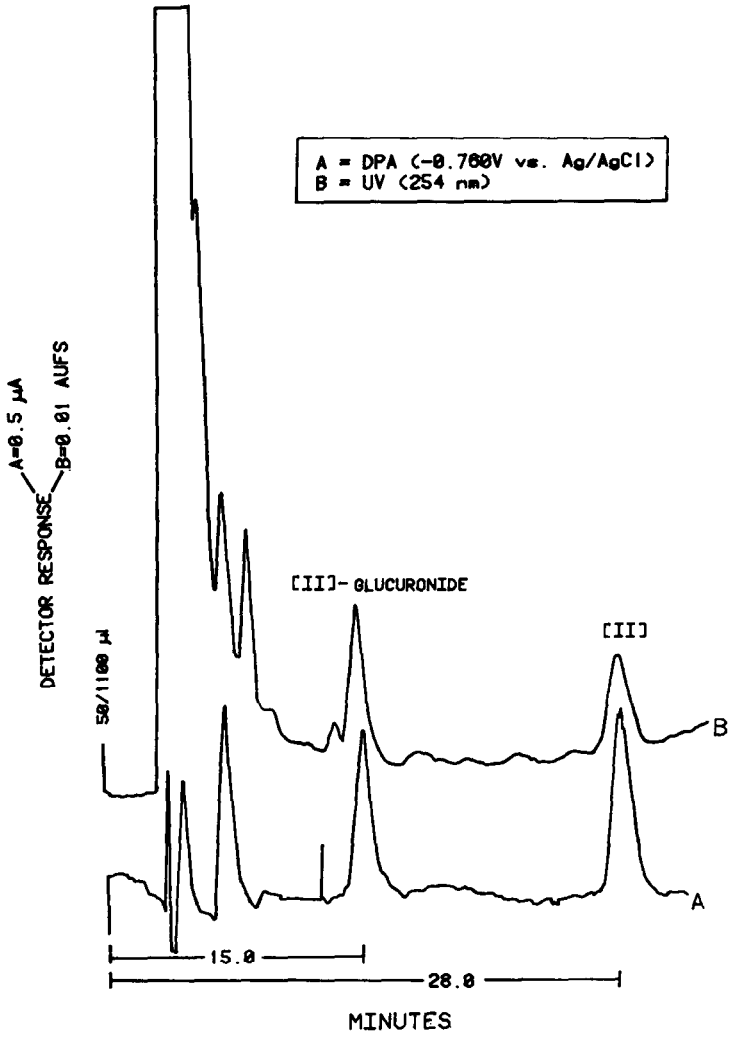


Fig. 11. Chromatograms of reversed-phase HPLC analysis of hydroxyethylflurazepam [II]; the major metabolite of flurazepam in the 1—2-h post-dose urine incubated for 30 min, monitored by tandem dectection using (a) differential pulse amperometry (DPA) and (b) UV at 254 nm. Column: μBondapak C_{18}; mobile phase: 0.0075 *M* acetate buffer (pH 3.5)—methanol (50 : 50); flow-rate: 0.9 ml/min at 100 bar.

the variety of modes of operation, i.e. reversed phase, normal phase adsorption, ion exchange, the variety of mobile phases and extensive library of column packings available for analysis. These factors coupled with the wide spectrum of detectors available [UV, fixed or variable wavelength, fluorescence, electrochemical (oxidative/reductive), and more recently, mass spectrometry interphased with LC as a viable detector], have advanced the utility of HPLC analysis considerably.

Although normal-phase HPLC is still popular, the limitations of the column packing (silica) and the organic mobile phases required restricts its utility. Reversed-phase HPLC has emerged as the predominant technique especially in

therapeutic drug monitoring, since all the usable detectors are compatible with the aqueous mobile phases used with the variety of column packings available.

Trace enrichment methods for drug analysis have been described for segmented Technicon® "Fast LC" systems [51] and involves organic solvent extraction, phase separation, an "evaporation to dryness" module which deposits the solvent sample residue on a moving PTFE belt from which the residue is redissolved in the mobile phase for HPLC analysis. Analysis of theophylline and four anticonvulsants, primidone, phenobarbital, phenytoin, carbamazepine and their active metabolites is performed in the system with UV detection at 270 nm.

In contrast to the segmented system is the direct analysis of the biological sample without solvent extraction by employing column-switching techniques to effect trace enrichment of the analyte on a precolumn prior to elution onto the analytical column for eventual quantitation. The sample is introduced onto the precolumn which retains the analyte, flushing the lipids out to waste (Fig. 12a). After flushing the precolumn to further clean up the sample (Fig. 12b),

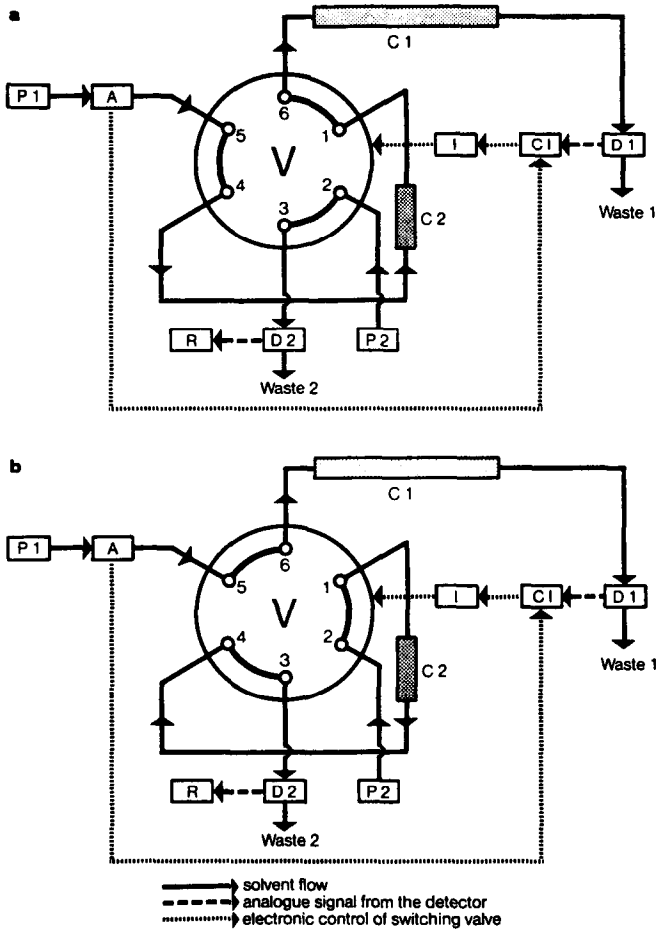


Fig. 12. Flow diagram illustrating column switching techniques used for trace analyte enrichment in the fully automated analysis of drugs and their metabolites in biological fluids. P1 = pump 1; P2 = pump 2; V = switching valve; C1 = column 1; C2 = column 2; D1 = detector 1; D2 = detector 2; Cl = controller; I = integrator; R = recorder; A = AD converter.

the analyte is back flushed or stripped from the precolumn and moved onto the analytical column (Fig. 12a), for eventual quantitation using either UV, fluorescence or electrochemical detection. The entire process is under microprocessor control from initial sample introduction to final data reduction and reporting, hence represents a truly automated system.

A fully automated HPLC system for drug monitoring by direct injection of plasma, saliva or urine was first described by Roth and Beschke [52]. The system consists of a programmable automatic sampling WISP® unit connected via two alternating precolumns for sample enrichment into the analytical column (all containing reversed-phase C_{18} packing) for eventual quantitation by fluorometric detection. The system was reported to have high precision (coefficient of variation, C.V. = 1.4%), and excellent stability with an inter-assay precision ranging from 3 to 5% (C.V.). The volume of biological sample injected varied from 10 to 150 μl depending on the medium used. The chromatograms obtained are extremely "clean" containing only trace amounts of endogenous compounds in addition to the analyte(s) which predominates in the analytical "midcut" processed. The system offers the advantages of minimal sample handling, combined with automated 24-h unattended operation for high sample throughput. Automated sample clean-up in HPLC using column-switching techniques was also recently reviewed [53].

HPLC analysis using post-column reaction detection is another viable means of quantitation of an analyte with enhanced sensitivity and specificity. The automated determination of secoverine in blood after enzymatic hydrolysis with subtilisin-A to release protein-bound drug was described [54], using precolumn switching for analyte enrichment followed by post-column paired-ion reaction with dimethoxyanthracene sulfonate for fluorometric determination. The system could be readily adapted for the quantitation of glucuronide—sulfate conjugates of drugs in urine as their aglycones.

Post-column detection by photochemical reaction by UV irradiation to yield fluorescent products is another novel application described [55]. A photochemical reactor using a medium- or high-pressure arc lamp (Hg, Xe or Xe—Hg) as the UV source was used to convert a variety of drug analytes; e.g., vitamin K, clobazam and its desmethyl metabolite, demoxepam, and phenothiazenes (mesoridazine, thioridazine, sulforidazine) into photochemically irradiated fluorescent products for quantitation with nanogram sensitivity. Post-column derivatization techniques available for HPLC analysis were also recently reviewed [56].

Microbore HPLC like its counterpart capillary GLC is very promising due to its high-resolution capacity which could be advantageous in the simultaneous analysis of several analytes (drugs and metabolites) with high precision and sensitivity. The relatively long analysis time of the order of 30—45 min per run is a disadvantage for high sample throughput. The utility of microbore HPLC in the analysis of bulk drugs and their breakdown products, isomers or homologues to advantage was recently demonstrated [57].

HPLC analysis in the fully automated mode coupled to very sensitive and specific detectors (UV, fluorescence or electrochemical) has reached a high degree of sophistication and complexity. The search for even more sensitive and specific detectors continues unabated. The new horizons yet to be fully

utilized for drug analysis in biological fluids lies in the fields of LC—MS using either electron impact (EI) or CI in both PICI and NICI modes [58,59], and derivative spectroscopy using multichannel diode-array UV—VIS and luminescence spectrophotometers as detectors for HPLC analysis for three-dimensional spectral characterization of new metabolites in biological fluids [60], was reviewed.

The use of tuneable dye lasers as excitation sources in fluorometric detection of drugs following HPLC separation is another novel technique for enhancing sensitivity and specificity of detection. The monochromatic laser energy is able to excite a significantly larger population of the analyte molecules present, thus enhancing sensitivity of detection. Problems in cell geometry and minimizing light scatter and interference have been a major drawback. The potential of the technique was demonstrated for aflatoxins, adriamycin and daunorubicin, two antitumor agents, and vitamins such as riboflavin which have been detected with picogram sensitivity [61].

10. CONCLUSION

The chemical structure and the pharmacokinetics of a compound influence not only the sensitivity and specificity requirements of the assay, but also the most suitable biological specimen for its quantitation. The criteria to be used in sample preparation and the analytical method selected for quantitation should aim to optimize all of the above factors in the eventual development of a reliable and validated method for the compound suitable for use in therapeutic drug monitoring [62].

11. ACKNOWLEDGEMENTS

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12. SUMMARY

Therapeutic drug monitoring can involve quantitation in either microgram, nanogram or picogram concentrations present in a complex biological matrix (whole blood, urine or tissue).

The chemical structure of a compound influences not only the analytical method best suited to its quantitation, but also its acid/base character (pK_a) and its extractability. The dose administered, the bioavailability of the dosage form, and the pharmacokinetic profile of the drug govern the circulating concentrations of either the parent drug and/or its metabolites present in vivo, and dictate the ultimate sensitivity and specificity required of the analytical method.

The degree of sample preparation required is dependent on the analytical method used (gas—liquid chromatography, thin-layer chromatography, high-performance liquid chromatography) and on the tolerance of the specific type of detection system to contamination. Factors leading to compound losses during sample preparation (adsorption, stability) are critical at low

concentrations and can adversely affect the reliability of an assay, therefore maximizing the overall recovery of the assay is essential not only for high sensitivity but also for good precision and accuracy. Therefore, the criteria to be used in sample preparation should aim to optimize all of the above factors in the overall development of a reliable and validated method for the compound suitable for use in clinical therapeutic monitoring.

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