Pure & Appl. Chem., Vol. 55, No. 12, pp. 1905-1924, 1983.
Printed in Great Britain

0033-4545/83 $3.00+0.00
Pergamon Press Ltd.

MICROANALYSIS OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

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Abstract - 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 clinical therapeutic monitoring.

Introduction

Therapuetic drug monitoring can involve quantitation in the microgram (10^{-6} gm or ppm), nanogram (10^{-9} gm or ppb) or picogram (10^{-12} gm or ppt) concentration range. These concentrations are present in a complex biological matrix (whole blood/urine/tissue) from which it must be selectively extracted and "cleaned up" prior to quantitation, Schill, (1).

The chemical structure of a compound influences the analytical method best suited to its quantitation, while the ionizable groups in the molecule determine its acid/base character (pKa), and its extractability, de Silva (2). The dose administered (mg/kg), the bioavailability of the dosage form, and the pharmacokinetic profile of the drug govern the absolute concentrations of the parent drug and/or metabolites to be quantitated, Kaplan and Jack (3), Schwartz and de Silva (4). These criteria influence the ultimate sensitivity and specificity required of the analytical method, de Silva (5), and the type of biological medium best suited for analysis, i.e. whole blood, plasma or urine, de Silva (6).

The degree of "clean up" required is dependent on the analytical method used (GLC, TLC, 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 analytical 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 adversly affect the reliability of an assay. Consequently, maximizing the overall recovery of the assay is essential not only for sensitivity but also for good precision and accuracy.

The armentarium of sensitive and specific methods currently available to the analyst is quite diverse and covers a wide linear dynamic range for quantitation (Figure 1).

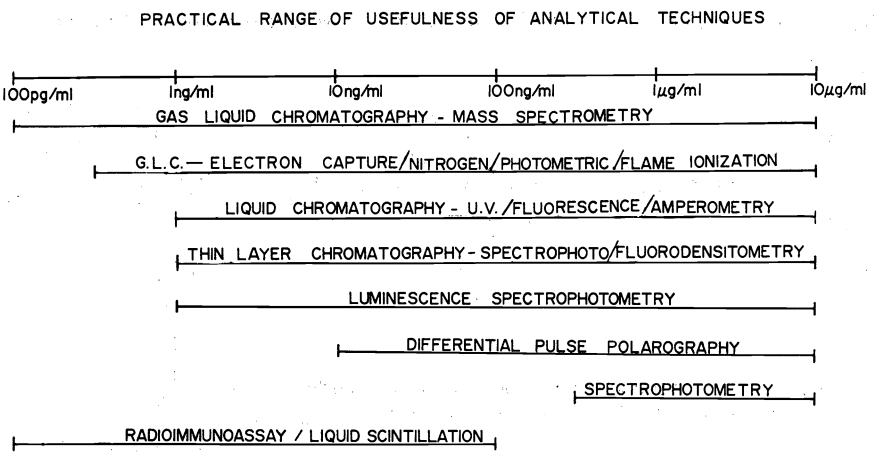


Fig 1. Practical range of usefulness of analytical techniques.

These include chromatographic techniques with a variety of selective detectors to ensure specificity [e.g. GLC with ionization detectors such as electron capture (ECD), nitrogen/phosphorus specific detector (N/P-D), mass spectrometric-chemical ionization detectors (GC-CI-MS), HPLC with U.V., fluorescence and electrochemical detectors (oxidative and reductive-polarographic), HPTLC - high performance thin layer chromatography 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, de Silva, (2).

I. Chemical Structure and Analytical Utility

Chemical manipulation of a drug via derivatization enhances the sensitivity and the specificity of the assay, Lawrence and Frei (7), Blau and King (8), and has certain advantages even if the intrinsic sensitivty 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 aliquot of the final residue analyzed can be reduced by sample dilution, significantly improving chromatographic analysis by minimizing endogenous interferences, resulting in more acurate, reproducible and reliable quantitation. All of the above factors should be optimized in the overall development of a reliable and validated method for eventual therapeutic drug monitoring.

The intrinsic properties of the 1,4-benzodiazepines/2-ones and imidazobenzodiazepines enables quantitation by EC-GC or GC-CI-MS due to the electro-negative groups (7 Halo or Nitro) and the amide function (>N_1-C_2(=O)-) in the diazepine ring and by HPLC using either UV (254 nm) or electrochemical detection by differential pulse polarography of the azomethine (>C_5= N_4 -) group in the molecule, de Silva (9).

Imidazo-1,4-benzodiazepine · carboxamide, a potent anxiolytic agent is administered in doses as low as 0.1-0.2 mg/70 kg adult. Direct analysis by EC-GLC was unsuccessful due to its high m.p. > 350° and resulted in broad peaks with poor sensitivity. A sensitive and specific GC-NCI-MS assay was developed by converting the compound into a volatile nitrile derivative by reacting the amide

group with pentafluoropropionic anhydride in trimethylamine with a sensitivity of 100 pg/ml of plasma (Figure 2).

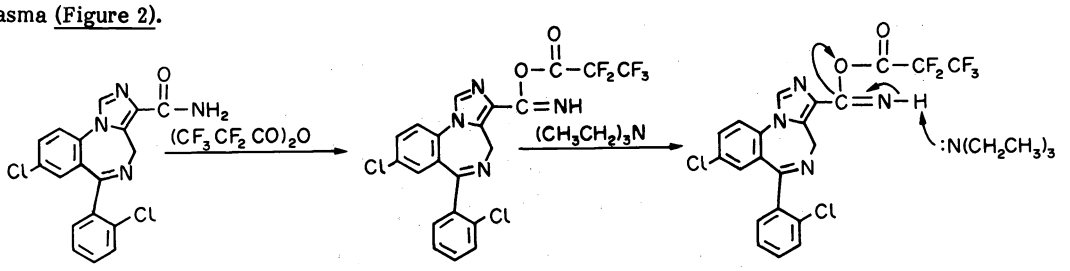
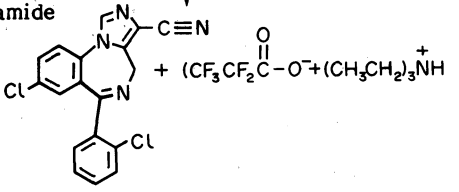
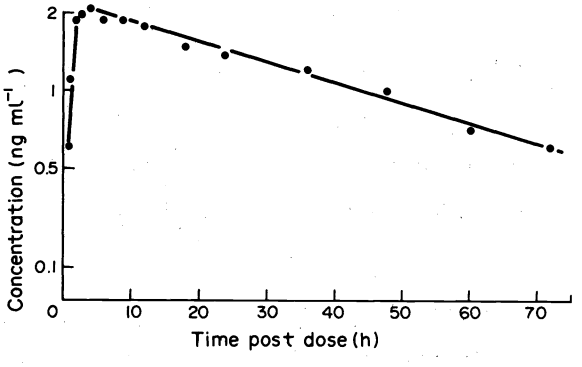


Fig. 2. Conversion of Imidazobenzodiazepine-3-Carboxamide to a Nitrile derivative for GC-NCI-MS analysis.



Plasma concentrations in man following a 0.2 mg oral dose showed peak concentrations of 2 ng/ml declining to 600 pg/ml at 70 hrs (Figure 3), documenting the clinical utility of the assay, Rubio et al (10).

Fig. 3. Plasma concentration-time curve in man following a single 0.2 mg oral dose of Imidazobenzodiazepine-3-Carboxamide



Guanidine compounds are difficult to analyze at low concentrations due to their high polarity. Guanfacine, a new antihypertensive agent and its 3-hydroxy metabolite were analyzed by EC-GLC with high sensitivity by reaction of the guanidine group with hexafluororacetyl acetone to form a triazine derivative. The NH and OH groups in the molecule were methylated with CH_3I by extractive alkylation (Figure 4).

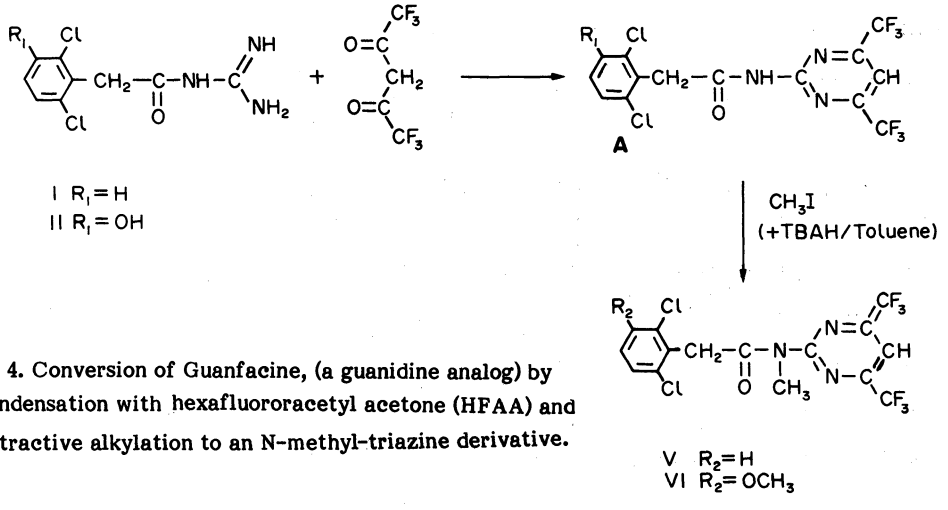


Fig 4. Conversion of Guanfacine, (a guanidine analog) by condensation with hexafluororacetyl acetone (HFAA) and extractive alkylation to an N-methyl-triazine derivative.

This volatile complex derivative was determined in urine by EC-GLC with good sensitivity (25 ng/ml). A comparison of chromatography using packed vs. capillary columns is shown in Figure 5, Guerret et al (11).

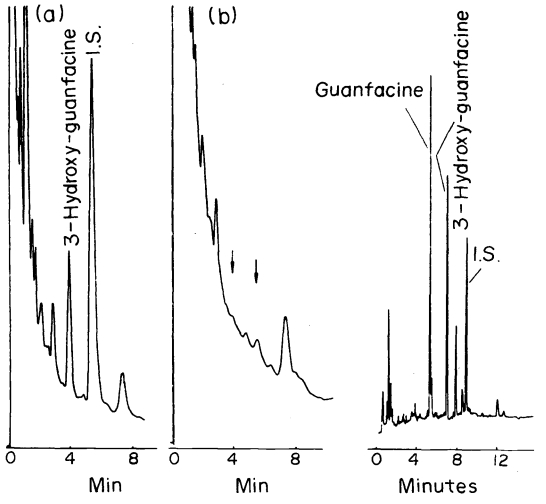


Fig 5. Chromatograms (EC-GLC) of a 0-24 hr urine extract from a subject given a 2 mg oral dose of Guanfacine a) patient sample extract, b) patient "control" sample both on a packed column and c) patient sample extract on a capillary column.

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. GC-NCI-MS analysis of the drug as its-OTMS, Pentafluorobenzyl ester enabled its sensitive and specific quantitation with a limit of 100 pg/ml of plasma, Min et al (12). Endogenous prostaglandins have likewise been determined using both capillary EC-GLC and capillary GC-MS analysis, Chiabrando et al (13).

III. Pharmacokinetic Factors Which Influence Sample Preparation

A. Biotransformation

Metabolic studies on the "in vitro" biotransformation of a compound using microsomal (9000 x g) enzyme preparations and/or "in vivo" studies using radioisotopically labelled (^{14}C, ^{3}H) compound should be underway in parallel with method development.

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 GC-MS, NMR and the synthesis of authentic reference compounds will enable their quantitation in biological fluids using sensitive and specific chemical methods, Gorrod and Beckett (14).

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.

B. 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, (14). The concentration of a predominant metabolite is usually sufficiently high to warrant its analysis in urine as in bioavailability/bioequivalence studies for dosage form evaluation, de Silva et.al. (15).

IV. 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, Shang-Qiang and Evanson (16).

V. Sample Processing vs Analytical Determinate Step

A. Analysis of blood/plasma/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/tissue homogenate or proteolytic digest are outlined in Figure 6.

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, de Silva, (17).

B. Analysis of Urine and Feces

A flow diagram for the extraction of urine and feces is outlined in Figure 7.

The analysis of these two media requires an aliquot of a representative sample, i.e., an aliquot from a total voidance collected over a known excretion period (e.g., 24, 48 hrs.). 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

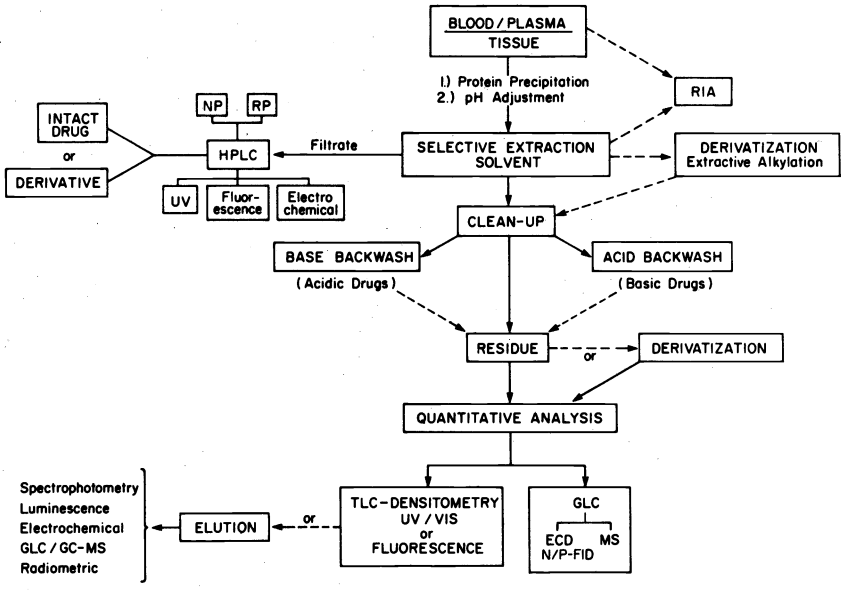


Fig 6. Flow diagram of the analytical options available for processing blood/plasma/tissue homogenate.

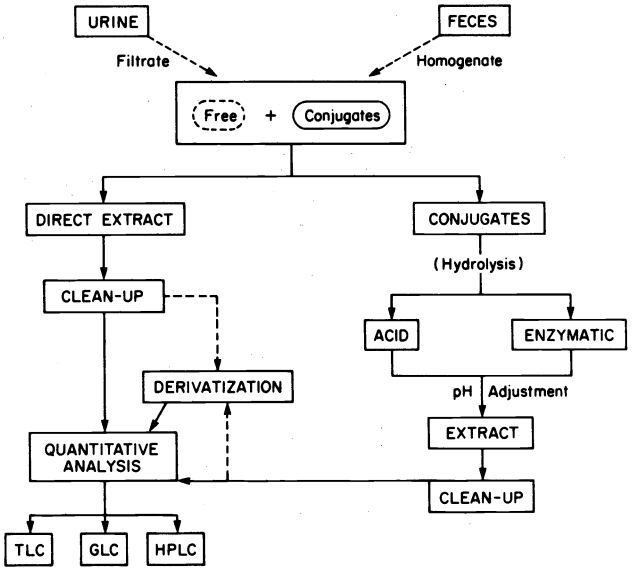


Fig 7. Sample preparation of urine and feces for the extraction of drugs and metabolite conjugates.

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 is hydrolyzed either with acid to cleave hippurates and other amino acid conjugates or incubated with β-glucuronidase/sufatase at 37°C for 2-12 hrs in a Dubnoff Incubation shaker to cleave glucuronide/sulfate conjugates. The aglycone/s are extracted after appropriate pH adjustment, followed by "clean up" of the extract either by chromatography (column or TLC) or liquid-liquid partition (acid/base).

Depending on the determinate step, the residue of the final extract (blood, plasma, tissue, urine or feces) may have to be derivatized (silylation of hydroxyl groups, esterification of carboxylic acids, etc.) for GLC analysis, or analyzed per se by either TLC densitometry or HPLC (usually reverse phase) using U.V., fluorescence or elecrochemical detection. HPLC has become the method of choice because non-volatile or thermally labile components can usually be analyzed without derivatization and where usually 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 by Midha and Hawes (18).

C. Extraction of the analyte from a biological matrix

Whole blood, plasma or serum is the biological specimen usually analyzed for theapeutic 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, GC-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, Frazer et al (19).

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 precipatation 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 (pff) 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 adsorbant cartridges containing a plug of reverse phase (C_{18}) HPLC packing material has found commerical 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 > 90%. The extracts were analyzed by reverse phase HPLC and correlated well against GLC and EMIT analysis. A variety of adsorbant cartridges (e.g. ion exchange resins, reverse phase (C_{18}) packing), makes it a viable procedure, Williams and Viola (20).

Solid extraction columns containing C_{18} packings (e.g. Bond Elut® from Analytichem International, USA) contained in polyethylene hypodermic syringes with luerlock fittings (1 ml capaicty) are convenient for batchwise manual use. Small sample volumes (< 500µl) 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 25-50µl of mobile phase for reversed phase HPLC analysis. Benzodiazepines have been sucessfully analyzed with > 90% recovery making the technique suitable for use where small sample volumes are required, Good and Andrews (21), Rao et al (22).

The next step in automating sample handling procedures would have to be the use of robots interphasing sample preparation with analytical quantitation, and data reduction and documentation, Little (23).

VI. Non-Chromatographic (direct) Analytical Techniques

A. Absorptiometric and Luminescence Methods

Spectrophotemetric (UV-VIS) and luminescence emission (fluorescence and phosphorescence) analysis, de Silva (24), have been the classical methods used extensively in drug analysis. They possess good 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 (1) or solid extraction (21, 22), and/or a chromatographic separation step eg GLC-photometric detection, TLC or HPLC.

B. Differential Pulse Polarography (DPP)

Electrochemical methods have better specificity by virtue of the functional group(s) in the molecule involved, de Silva and Brooks (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 , Brooks (26). The DPP analysis of bromazepam [I], (an antianxiolytic agent) and its major metabolites in urine, viz, 3-hydroxy bromazepam [II] and 2-amino-3-hydroxy-5-bromo-benzoylpyridine [V] present mainly as glucuronide-sulfate conjugates, first involved selective extraction of the unconjugated intact drug [I] and 2-amino-5-bromobenzoyl-pyridine [IV] from conjugated [II] and [V], subsequently extracted as their aglycones after glucuronidase incubation.

The respective extracts were analyzed directly by DPP in 1.0 M phosphate buffer (pH5.5) which yielded two distinct peaks resulting from the reduction of the azomethine (>C_5=N_4-) group of the benzodiazepin-2-one and the carbonyl (>C=O) group of the benzoylpyridine component in each fraction, Figure 8.

This fortuitous set of conditions enabled the development of a specific assay without a chromatographic separation step utilizing selective extraction and the different functional groups involved in the two compounds to analytical advantage.

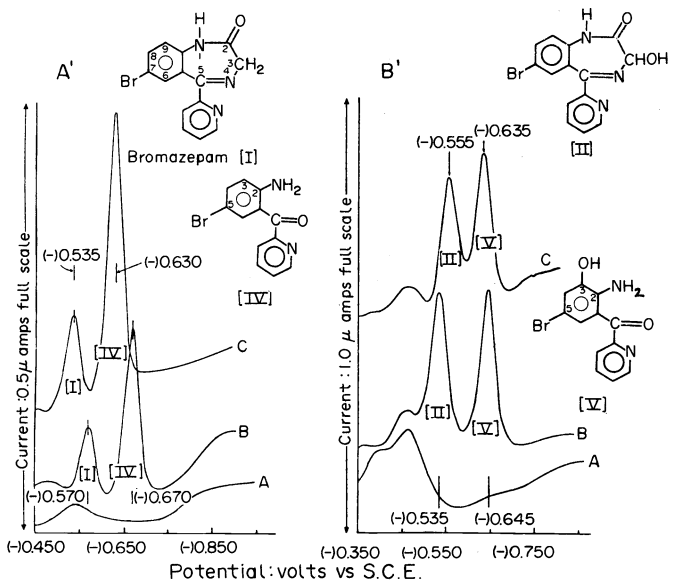


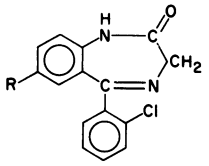
Fig 8. Differential pulse polarograms of A' bromazepam [I], and the amino-bromo-benzoylpyridine metabolite [IV] and B' the 3-hydroxy metabolites [II] and [V] obtained in 1.0 M pH 5.5 phoshate buffer as the supporting electrolyte (A) Control urine blank, (B) authentic standard mixture, (C) authentic compounds recovered from urine

C. Immunological Assays:

1. Radioimmunoassay (RIA) is especially useful in monitoring drugs in pediatric 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 as shown in Figure 9 for clonazepam.

Radioimmunoassays for benzodiazepines, Dixon (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. Dixon and Crews (28) described a novel method for the analysis of diazepam in 10 μl samples of blood or plasma and 100 μl samples of saliva



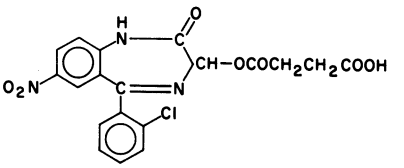
CLONAZEPAM

R

NO_2 = Clonazepam

NH_2 = 7-Amino-clonazepam

CH_3CONH = 7-Acetylamino-clonazepam



HAPTEN

7-Nitro-5-(2'-chlorophenyl)-2H-1,4-benzodiazepin-2-one-3-oxy-hemi succinic acid

Fig 9. Chemical structure of a specific hapten synthesized for the radioimmunoassay of clonazepam

following oral administration of a single 5 mg dose of Valium®. 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 was 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.

2. Radioreceptor Assays (RRA)

This 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 by Dubnick et al (29). Radioreceptor assays for specific benzodiazepines have also been reported by Skolnick et al (30), and are being used to quantitate the total "active" fraction of parent drug and pharmacologically active metabolites in pharmacodynamic studies.

VII. Chromatographic Techniques

A. Analysis by GLC

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 detectors such as the ECD and the N/P-D have to be considered during sample preparation so as not to introduce contaminants into the extract which could be detrimental.

The N/P selective detector, 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.

Where high sensitivity and resolution is required, GLC analysis using either the electron capture (ECD) or Nitrogen Specific (N/P-D) detector in conjunction with fused silica capillary columns is the "state of the art" in GLC analysis, and with GC-MS/SIM 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 the N/P-D 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 N/P-D is stable even when the electrometer is operated at maximum sensitivity, as reported by van Brunt (31).

EC-GLC analysis has been extensively used in the determination of the 1,4-benzodiazepines (which are widely used in clinical practice as antianxiolytics, muscle relaxants, hypnotics, and anticonvulsant agents), Costa (32), due to the presence of an electronegative group in the 7 position of the molecule (usually a halogen or nitro group), de Silva (11). 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 EC detector 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, Figure 10.

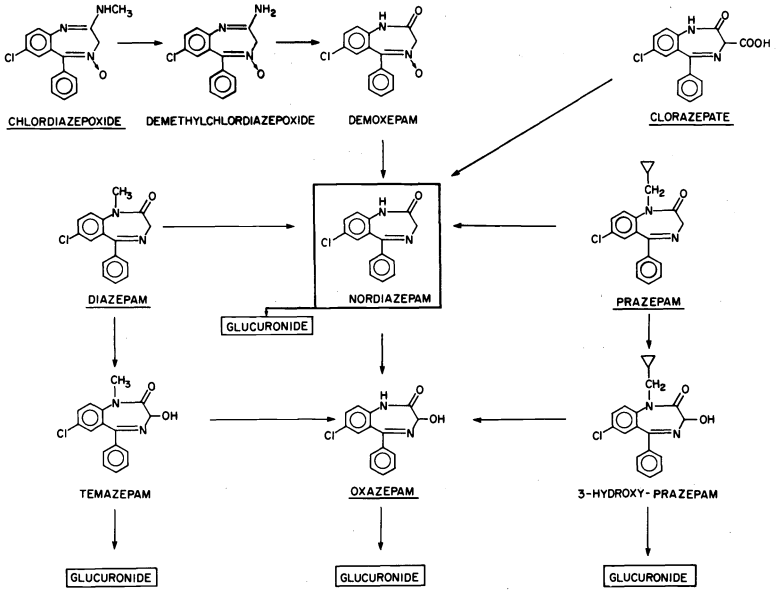


Fig 10. Biotransformation of benzodiazepines to metabolites common to several drugs

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. A review of EC-GLC methods for the determination of drugs and metabolites was reported by de Silva and Puglisi (33).

The properties of a drug molecule that render it amenable to EC-GLC analysis can be readily adapted to the development of highly sensitive and specific GC-Chemical Ionization-MS assays operated in either the positive ion (PI) or negative ion (NI) modes of analysis. The benzodiazepines lend themselves excellently to GC-CI-MS analysis, Garland and Miwa (34).

B. Gas Chromatography-Chemical Ionization-Mass Spectrometry (GC-CI-MS)

Chemical ionization GC-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 specifitiy of analysis, Garland and Powell (35).

GC-NCI-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 chemical ionization (PCI-MS) methods. This was demonstrated for the 7-nitro anticonvulsant, clonzepam which was analyzed by both GC-PCI-MS, Min and Garland (36), and GC-NCI-MS, Garland and Min (37). The $[M-H]^-$ ion monitored at m/z 314 using the ^{15}N, ^{18}O, stable isotope analog as the internal standard (m/z 321), (Figure 11), yielded a sensitivity limit of 100 pg/ml in the NCI mode compared to that of 1000 pg/ml in the PCI mode monitoring the $[MH]^+$ ion at m/z 316.

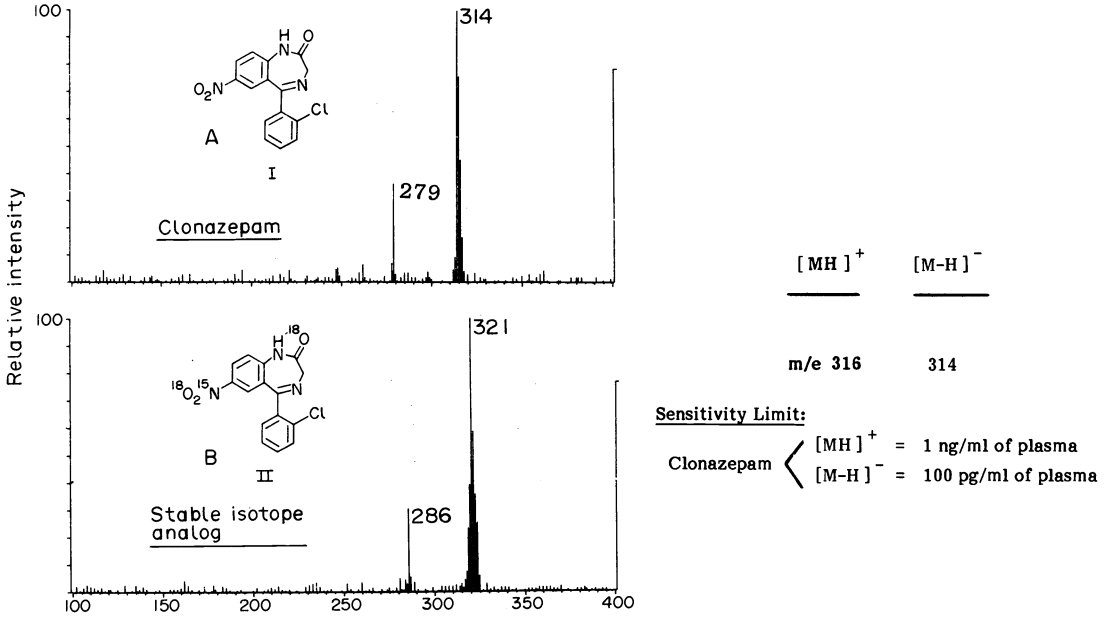


Fig 11. Methane GC-CI-MS analysis of clonazepam; a 7-nitro-1,4-benzodiazepin-2-one using positive ion (PI) and negative ion (NI) modes.

The sucess of GC-NCI-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 (10^{-12}g) sensitivity, Hunt and Crow (38).

C. Analysis by Thin Layer Chromatography

Thin layer chromatographic (TLC) analysis enables rapid devlopment of chromatographic parameters for eventual use in HPLC analysis. Preliminary separation of a drug/metabolites using radiolabelled compound enables qualitative identification via radiochromatographic scanning or autoradiography to isolate metabolites from either an "in vitro" 9000 x g microsomal incubation or from "in vivo" biotransformation studies (14). The separated compounds can be eluted from the silica gel and analyzed by a variety of selective techniques, e.g.,spectrophoto/fluorometry, polarography, GLC, GC-MS, NMR, not only for quantitation but also for structure elucidation purposes.

Quantitative analysis by "in situ" spectrophoto/flurometry has been extensively utilized, especially since the advent of high performance TLC using small sample aliquots 1 µl or less applied to the chromatoplate, rapid development and densitometric analysis, Fenimore (39). 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. These were extracted, separated by TLC and quantitated by "in situ"spectrofluorodensitometry (Figure 12), and applied to the determination of plasma concentrations of flurazepam [I] and its major metabolites; hydroxyethyl [II] and N-desalkyl [III], following a single 30 mg oral dose of Dalmane®[I]·2HCL, de Silva et al (40).

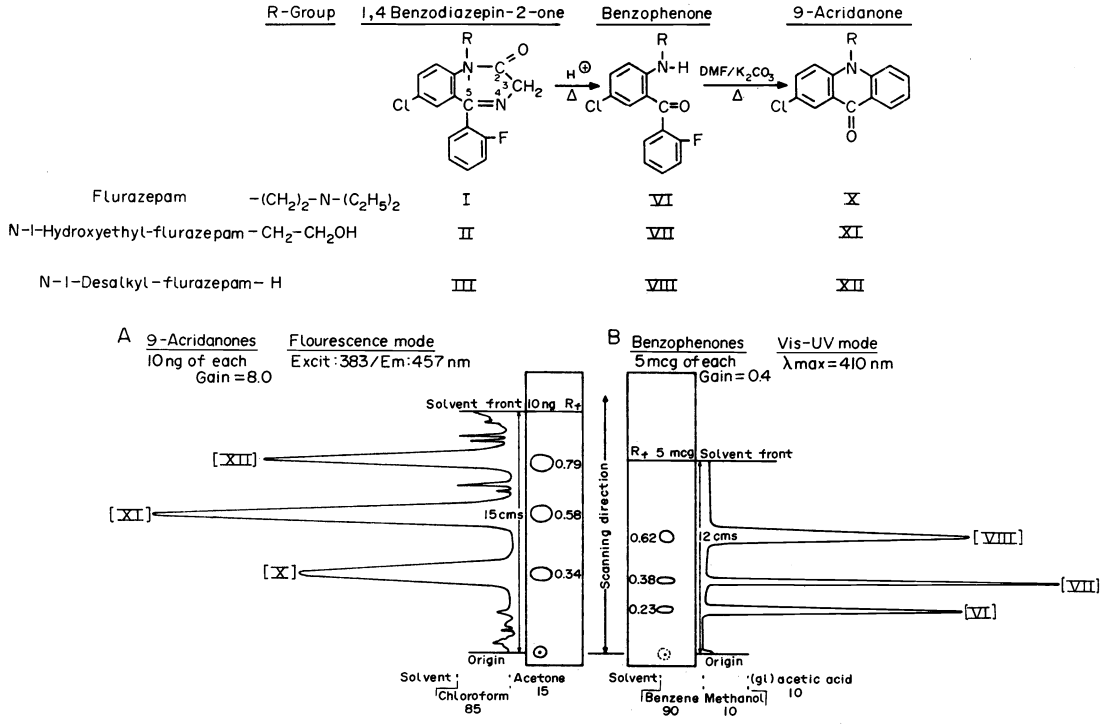


Fig 12. TLC-fluorodensitometric analysis of flurazepam [I] and its major metabolites [II] and [III] as their benzophenone or 9-acridanone derivatives

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.

D. Analysis by High Performance Liquid Chromatography (HPLC)

HPLC analysis has several advantages that can be collectively optimized for sensitive and specific detection. Two modes of operation, normal phase (adsorption) and reverse 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 quarternary (NH_3^+) compounds. Drug molecules can be analyzed either as the intact moiety or as a suitable derivative using either U.V., 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 (pff) (after partitioning with n-hexane as a clean up step to remove colloidal lipids), Blanchard (41). Urine is filtered to remove salts and colloidal materials, and an aliquot diluted in the mobile phase and analyzed directly using reverse phase HPLC.

Amoxicillin; [a β lactam antibiotic, (pKa= 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, Lee et al (42), (Figure 13).

Analysis in the fluorescence mode (Exit: 385/Emiss: 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.

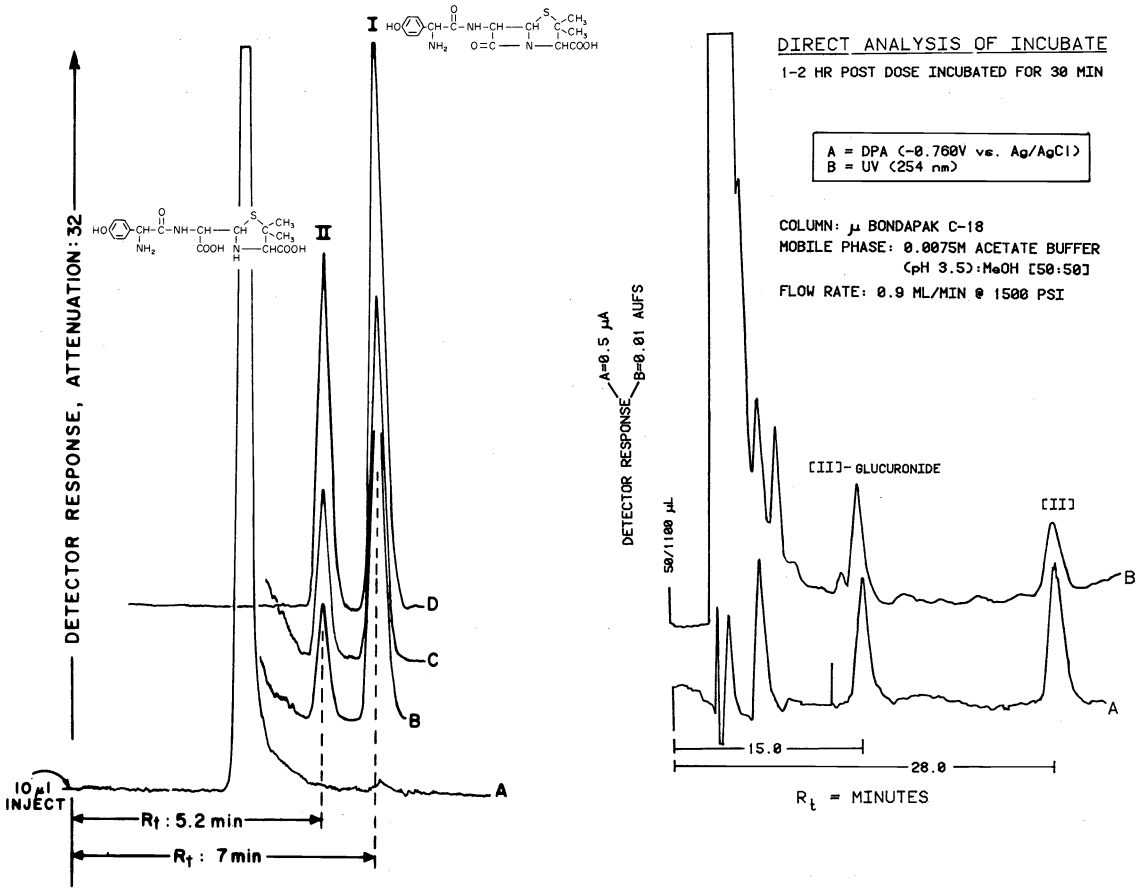


Fig 13. Chromtagorams of the HPLC analysis of amoxicillin [I] and its benzylpenicilloic acid [II] in human urine by fluorometric detection following postcolumn derivatization with fluorescamine: A= diluted control urine; B= post oral dose (250 mg); C = control urine with added authentic standards; D= authentic standards

Fig 14. Chromatograms of reverse phase HPLC analysis of the 1-2 hr post-dose urine incubated for 30 minutes, monitored by tandem detection by (a) differential pulse amperometry (DPA) and (b) by UV at 254 nm

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), Hackman and Brooks (43) using the functional group specificity of the [$>C_5=N_4-$] azomethine group attests to the utility of this technique.

Tandem detection for HPLC analysis can also be used to advantage in the elucidation of the kinetics of β-glucuronidase enzymatic hydrolysis of a major (key) metabolite in urine and its specific quantitation as the aglycone. The major urinary metabolite of flurazepam dihydrochloride, [I] · 2HCL, Dalmane® is N_1-hydroxyethyl flurazepam [II] -glucuronide which accounts for 30-55% of an orally administered dose in a 72 hour excretion period, de Silva et al (15).

The tandem detection of the column effluent first by UV at 254 nm followed by Differential Pulse Amperometry (DPA) ensured specificity for the [II] - glucuronide peak (R_t = 15.0 minutes) and for [II] (aglycone) (R_t = 28 minutes), Figure 14.

The chromatogram monitored by DPA (Figure 14-A) shows two extra components (probably minor metabolites), which are otherwise masked in the solvent front of the chromatogram monitored by UV at 254 nm (Figure 14-B) further attesting to the utility of DPA as a specific HPLC detector for the reduction of the [$>C_5=N_4-$] azomethine group common to all, 1, 4-benzodiazepines, Brooks (26).

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, Grossman (44). The oxidative detector is very low cost compared to UV, fluorescence or polarographic detectors, and its sensitivity has advanced the field of catecholamine biochemistry significantly. Whereas EC-GLC or GC-MS analysis requires derivatization of the phenolic and amine groups using silylating and perfluoro acylating agents, HPLC analysis can achieve the same sensitivity of 5-10 pg/ml using the electrochemical detector in the phenolic group specific oxidative mode, Baker and Coutts (45). A variety of cell geometries used in solid state carbon electrodes (tubular, thin layer and rotating disc) and dropping mercury electrodes (DME) types have been reviewed by Hanekamp et al. (46).

HPLC analysis has made very significant inroads into therapeutic drug monitoring which was once the monopoly of GLC, TLC or direct spectrophometric methods. The popularity of the technique is due largely to its versatility in the variety of modes of operation, i.e. reversed phase (RP), normal phase adsorption (NP), ion exchange (IEX), the variety of mobile phases and extensive library of column packings available for analysis. These factors coupled with the wide spectrum of detectors available U.V.-fixed/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 available detectors are compatible with the aqueous mobile phases used with the variety of column packings available. Automatability of HPLC has reached a high degree of sophistication. In addition to its ability to analyze either organic solvent extracts or a protein free filtrate (pff), column switching techniques using "pre columns" placed before the analytical column has enabled direct injection of the biological sample (blood/plasma/serum/saliva/urine)directly on the analytical system. The sample is introduced onto the precolumn which retains the analyte, flushing the lipids out to waste. After flushing the precolumn to further clean up the sample, the analyte is back flushed or stripped from the precolumn and moved onto the analytical column for eventual quantitation using either U.V./fluorescence/electrochemical detection. The entire process is under microprocessor control from initial sample introduction to final data reduction/reporting, hence represents a truly fully automated system.

Trace enrichment methods for drug analysis have been described for segmented Technicon®"Fast LC" systems by Dolan et al (47) and involves organic solvent extraction, phase separation, an "evaporation to dryness (EDM) module which deposits the solvent sample residue on a moving teflon 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 U.V. monitoring 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. A fully automated HPLC system for drug monitoring by direct injection of plasma/saliva/urine was first described by Roth et al. (48). The system consists of a programmable automatic sampling WISP®unit connected via two alternating precolumns for sample enrichment into the analytical column (all containing reverse-phase C_{18}-packing) for eventual quantitation by fluorometric detection. The system was reported to have high precision (C.V. = 1.4%), and excellent stability with an inter-assay precision ranging from 3-5% (C.V). The volume of biological sample injected varied from 10-150 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 predominate in the analytical "midcut" processed. The system offers the advantages of minimal sample handling, combined with automated 24 hour unattended operation for high sample throughput.

Automated sample clean up in HPLC using column switching techniques was recently reviewed by Karger et al. (49).

HPLC analysis using post column reaction detection is another viable means of quantitation of an analyte with enhanced sensitivity and specificity. Werkhoven-Goewie et al. (50) described the automated determination of Secoverine in blood after enzymatic hydrolysis with subtilisin-A to release protein-bound drug using pre-column 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 U.V. irradiation to yield fluorescent products is another novel application described by Birks and Frei (51). A photochemical reactor using a medium or high pressure arc lamp (Hg, Xe or Xe-Hg) as the U.V. 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 recently reviewed by Stewart (52).

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/metabolites) with high precision and sensitivity. The relatively long analysis time of the order of 30-45 minutes/run is a disadvantage for high sample throughput. Tsuji and Binns (53) demonstrated the utility of microbore HPLC in the analysis of bulk drugs and their breakdown products/isomers/homologues to advantage.

HPLC analysis in the fully automated mode coupled to very sensitive and specific detectors (U.V./fluorescence/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 chemical ionization (CI) in both positive chemical ionization (PCI) and negative chemical ionization (NCI) modes, Kenyon et al. (53, 54) and derivative spectroscopy using multichannel diode array UV-visible and luminescence spectrophotometers as detectors for HPLC analysis for 3-D spectral characterization of new metabolites in biological fluids, Talmi (55) and Overzet et al. (56).

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 has been demonstrated for aflatoxins, adriamycin and daunorubicin, two antitumor agents, vitamins such as riboflavin which have been detected with picogram sensitivity, Yeung and Sepaniak (57).

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 clinical therapeutic monitoring, Sadee and Beelen (58).

Acknowledgements

The author is indebted to Ms. S. Christopher and Ms. C. Renner for the preparation of this manuscript; to Elsevier Scientific Publishers, Amsterdam, The Netherlands, for permission to reproduce Figures 2, 3, 4, 5, 10, 11; to Ellis Horwood Publishing Co., Chichester, U.K. for Figures 6, 7, 13; to J. Pharmaceutical Sciences, Washington, DC, for Figures 8, 9, 12, and Plenum Press, N.Y. for Figure 14, respectively.

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