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ANALYTICAL STRATEGIES FOR THERAPEUTIC MONITORING OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

PLENARY LECTURE

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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.

INTRODUCTION

Therapeutic drug monitoring can involve quantitation in the microgram (10^{-6} g or ppm), nanogram (10^{-9} g or ppb) or picogram (10^{-12} g 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 [1].

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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 (pK_a) and its extractability [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 [3, 4]. These criteria influence the ultimate sensitivity and specificity required of the analytical method [5].

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. 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 assay 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 render it compatible to quantitation by a specific method [6, 7]. 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 [8].

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 with a variety of selective detectors to ensure specificity [e.g., GLC with ionization detectors such as electron-capture (ECD), nitrogen–phosphorus specific detector (NPD), mass spectrometric–chemical ionization detectors (CI–MS), HPLC with UV, fluorescence and electrochemical detectors (oxidative and reductive-polarographic),

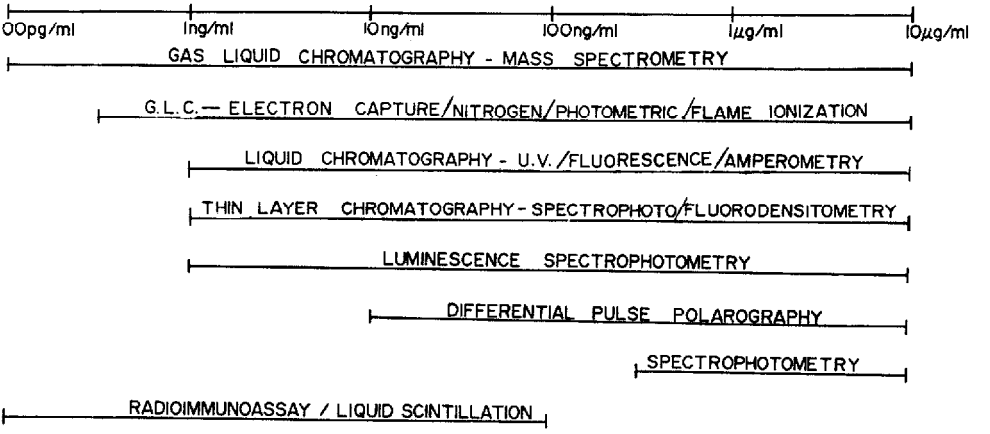


Fig. 1. Practical range of usefulness of analytical techniques.

high-performance thin-layer chromatography (HPTLC) with in situ spectrophotometry/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.

CHEMICAL STRUCTURE AND ANALYTICAL UTILITY

Chemical manipulation of a drug via derivatization is useful in enhancing both the sensitivity and the specificity of the determinate step [6, 7], 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 aliquot 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.

The development of a sequential series of assays is advantageous in developing methods with a wide range of useful sensitivity. Examples of two chemical derivatives used in the development of such a series of assays are shown in Fig. 2 for two nitroimidazoles. Either drug is first separated from metabolites by extraction followed by TLC and elution of intact drug from the silica gel with methanol. An aliquot of this eluate may be hydrolyzed in base to liberate nitrite which is used to diazotize sulfanilamide. This diazonium salt is further reacted to yield an azo-dye chromophore suitable for absorptiometric analysis with a sensitivity limit of about 1 μg/ml. The colored product is designated the Bratton—Marshall chromophore because this same diazotization reaction is the basis for the analysis of sulfanilamide and other sulfa drugs.

If greater sensitivity is required, another aliquot can be reacted to yield the trimethylsilyl (TMS) derivative which is sufficiently volatile to be analyzed by electron-capture GLC. This allows for an increase in sensitivity of three orders of magnitude.

Although not a derivatization procedure, DPP (Fig. 2), with about three times more sensitivity than that of the absorptiometric assay, is also available for these compounds.

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, 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.

Determination of the total radioactivity in plasma or urine vs. solvent-extractable radioactivity will indicate the extent to which polar non-extractable

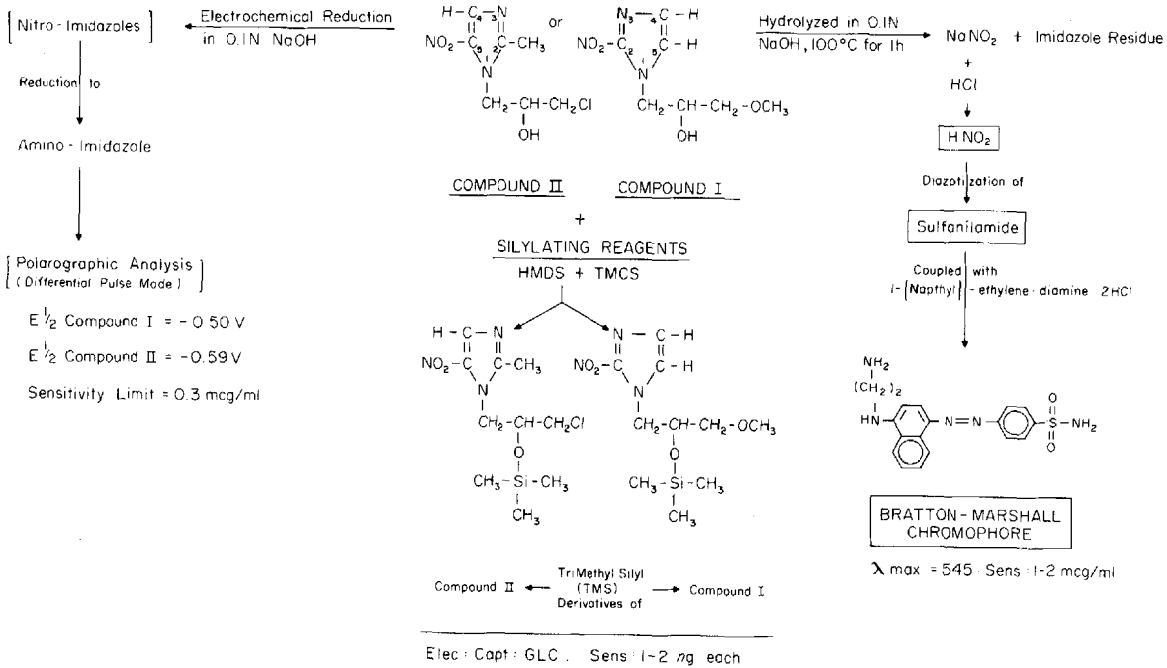


Fig. 2. Chemical reactions of a 2-nitro[I]- and a 5-nitro[II]imidazole used in multiple analytical methods.

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).

The plasma profile of ^{14}C-labelled clonazepam (anticonvulsant) in man (Fig. 3) indicated the presence of polar non-extractable metabolites when the total plasma ^{14}C-profile (upper curve) was compared to the diethyl ether extractable [^{14}C]-clonazepam profile (middle curve) in plasma. The excellent correlation between the clonazepam concentration in whole blood (lower dashed curve) determined by TLC—radiometry vs. EC—GLC verified the accuracy and specificity of the latter technique.

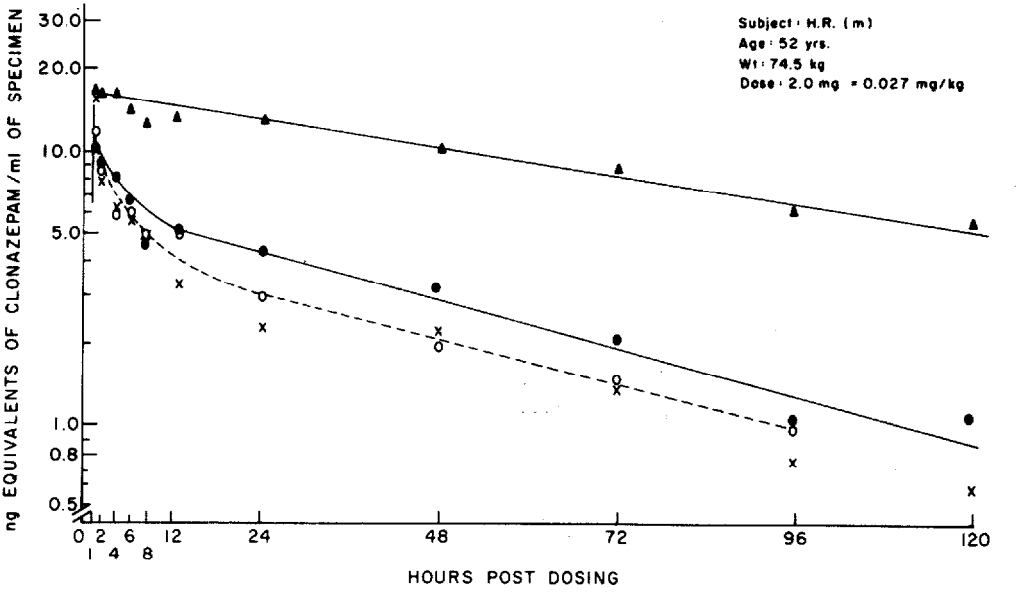


Fig. 3. Radiolabelled drug and its utility in the verification of assay sensitivity and specificity. Plasma levels of radioactivity and of intact clonazepam as a function of time following a 2-mg oral dose of micronized [^{14}C]clonazepam. Plasma total ^{14}C in ng equivalents of clonazepam per ml (▲), plasma [^{14}C]clonazepam in ng/ml (●), blood [^{14}C]clonazepam (×) by radiometry, and blood clonazepam determined by EC—GLC analysis (○) in ng/ml.

PHARMACOKINETIC FACTORS WHICH INFLUENCE SAMPLE PREPARATION

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 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.

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 [9]. Their concentrations are usually sufficiently high to warrant their analysis in urine as in bioavailability/bioequivalence studies for dosage form evaluation.

SAMPLE PROCESSING VERSUS ANALYTICAL DETERMINATE STEP

Analysis in 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 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 [8].

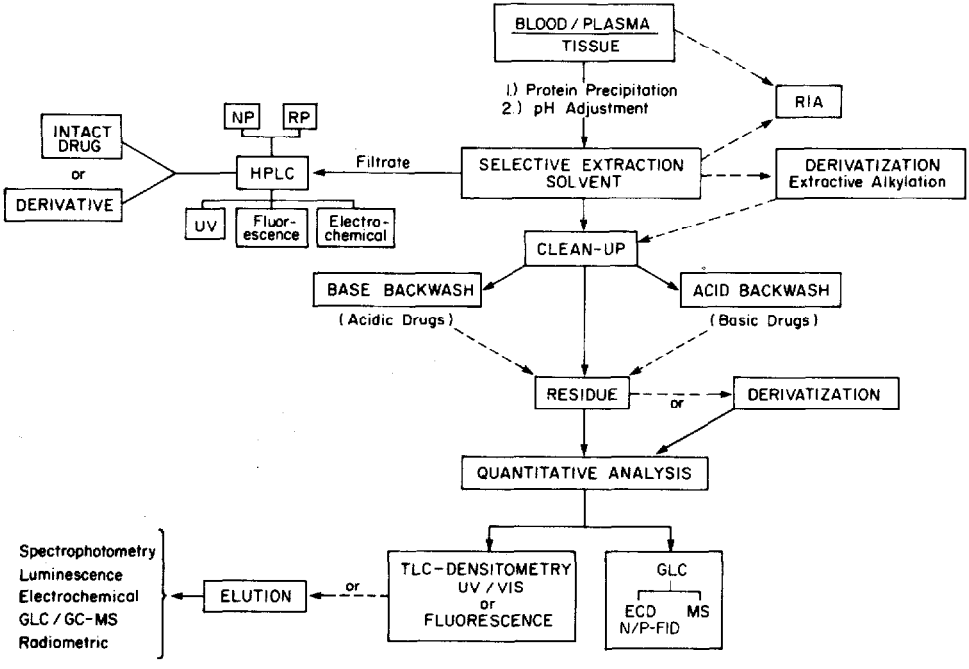


Fig. 4. Flow diagram of the analytical options available for sample processing.

Radioimmunoassay. RIA can be performed either directly in the biological sample (plasma or serum) or in the residue of the solvent extract which can also be used for HPLC analysis.

HPLC. Analysis by HPLC 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 ammonium compounds. 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/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 by GLC 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 detectors such as ECD, NPD or CI—MS.

TLC. Analysis by TLC may be performed directly on the residue of the initial solvent extract, unless prechromatographic 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 nonpolar 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).

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, 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" (directly extractable) and "bound" (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 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 may

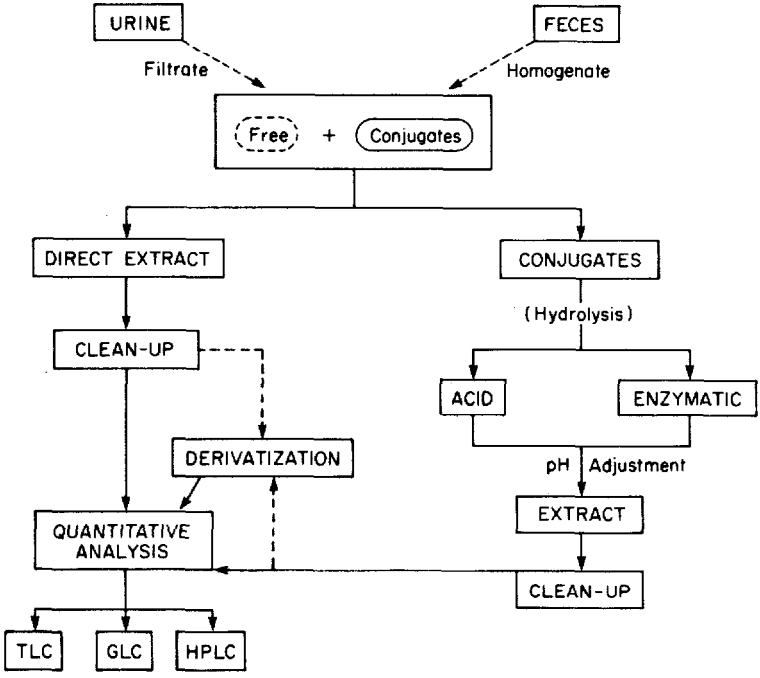


Fig. 5. Sample preparation of urine and feces for the extraction of drugs.

have to be derivatized (silylation of hydroxyl groups, esterification of carboxylic acids) for GLC analyses, 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 the components can usually be analyzed without derivatization and concentration is not limiting. Resolution of endogenous impurities not removed by previous clean-up may be a limiting factor.

CHROMATOGRAPHIC TECHNIQUES

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 NPD have to be considered during sample preparation so as not to introduce contaminants into the extract which could be detrimental.

The nitrogen—phosphorus 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 extracted. Thus, the selection of the proper type of syringe and collection tubes becomes 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.

GLC analysis has been extensively used in the determination of compounds of the 1,4-benzodiazepine class which has yielded pharmaceuticals which are widely used in clinical practice as antianxiolytics, muscle relaxants, hypnotics, and anticonvulsant agents [10]. The benzodiazepines undergo extensive biotransformation in man and other animal species usually resulting in the presence of one or more pharmacologically active metabolites [11], which have to be resolved from the parent drug for accurate quantitation in biological fluids (Fig. 6). Since the clinically effective 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.

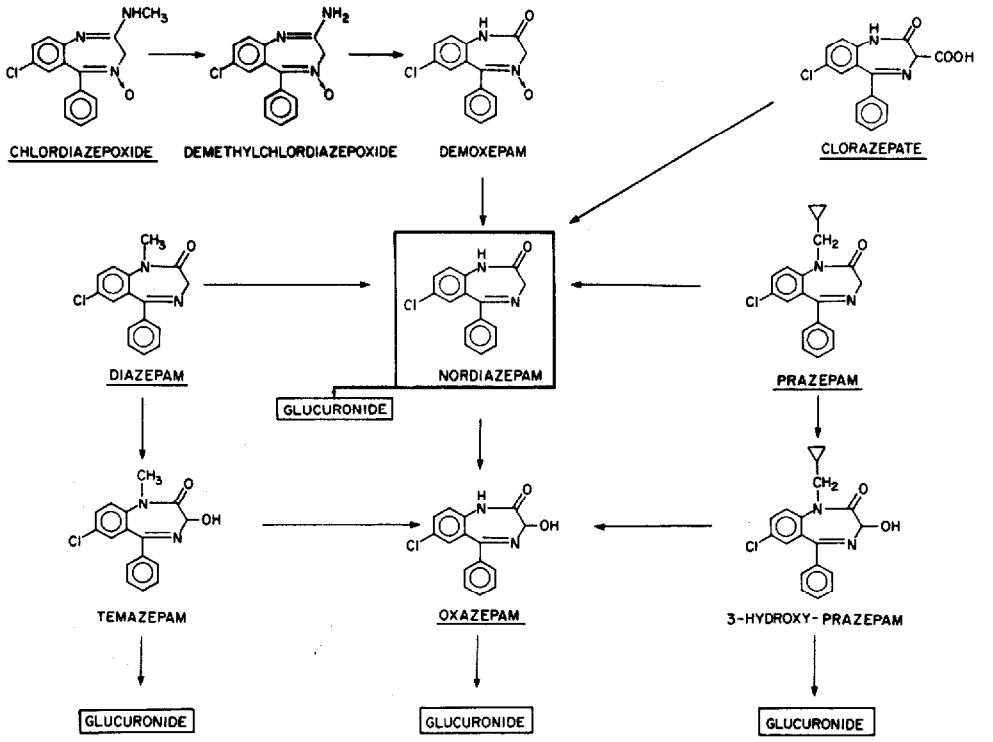


Fig. 6. Biotransformation of benzodiazepines to metabolites common to several drugs.

The majority of the GLC assays reported [12] for the determination of specific benzodiazepines in biological fluids use electron-capture detection due to the presence of an electronegative group in the 7 position of the molecule (usually a halogen or nitro group). 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.

Midazolam, [I], an imidazo-1,4-benzodiazepine (pK_a 1.7, 6.15) is currently being developed orally as short acting hypnotic and parenterally as a pre-operative anesthesia inducing agent administered by intravenous infusion. Clinically active doses are of the order of 10 mg per 70 kg (ca. 0.143 mg/kg). The compound undergoes extensive "first pass" metabolism by oxidation (Fig. 7),

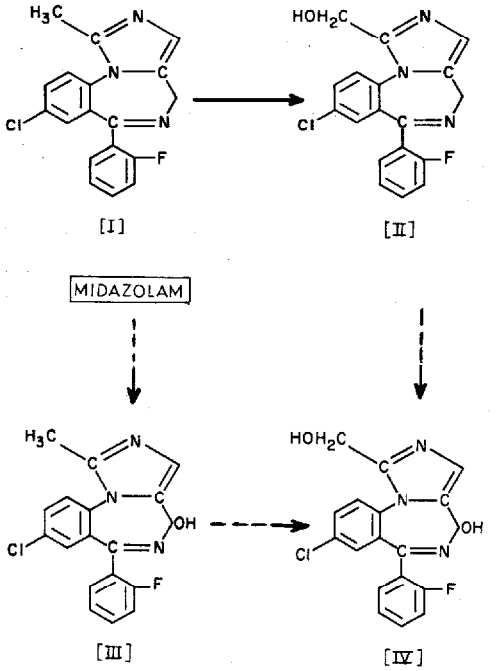


Fig. 7. Biotransformation of midazolam in man.

yielding the 1-hydroxymethyl analogue [II] as the major plasma metabolite which is eliminated as a glucuronide/sulfate conjugate.

EC—GLC analysis of plasma for [I]- and [II]-OTMS (Fig. 8) (sensitivity limit 5—10 ng/ml) enabled the elucidation of the pharmacokinetic profile of [I] and [II] in man. The elimination half-life ($t_{1/2}\beta$) of [I] and [II] is about 2 h, with a significant percentage of the dose eliminated in urine as [II]-glucuronide accounting for 40—45% following intravenous [13], and 60% of the dose following oral administration [14] in a 24- 48 h excretion interval. The major metabolite [II] is extracted from urine after glucuronidase incubation; the residue is silylated and analyzed by EC—GLC. The high sensitivity of [II]-OTMS to ECD enables sufficient sample dilution such that in the quantitation of [II]-OTMS, the bulk of the endogenous interfering peaks and the minor metabolites [III] and [IV] are diluted out ensuring its accurate quantitation (Fig. 9).

The properties of a drug molecule that render it amenable to EC—GLC analysis can be readily extended to the development of highly sensitive and specific GC—CI—MS assays obtained in either the positive ion (PI) or negative ion (NI) modes of analysis. The benzodiazepines lend themselves excellently to GC—CI—MS analysis [15].

Gas chromatography—chemical ionization—mass spectrometry

GC—CI—MS analysis is rapidly establishing itself as the method of choice 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 gener-

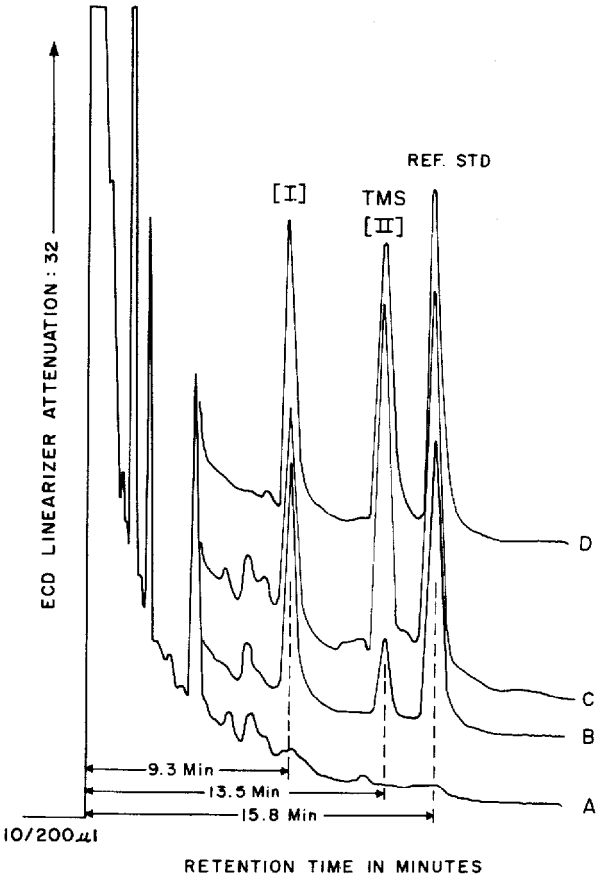


Fig. 8. Chromatograms of (A) control human blood extract; (B) control blood extract containing added authentic standards of midazolam [I] and 1-hydroxymethyl analogue [II]; (C) subject post-dose blood extract (10 mg i.v. infusion); and (D) authentic standards of compounds [I] and [II]-OTMS.

ated 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 [16].

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, clonazepam which was analyzed by both GC—PCI—MS [17] and GC—NCI—MS [18]. 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) (Fig. 10), yielded a sensitivity limit of 100 pg/ml in the NCI mode compared to 1000 pg/ml in the PCI mode monitoring the $[MH]^+$ ion at m/z 316.

The success 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, penta-

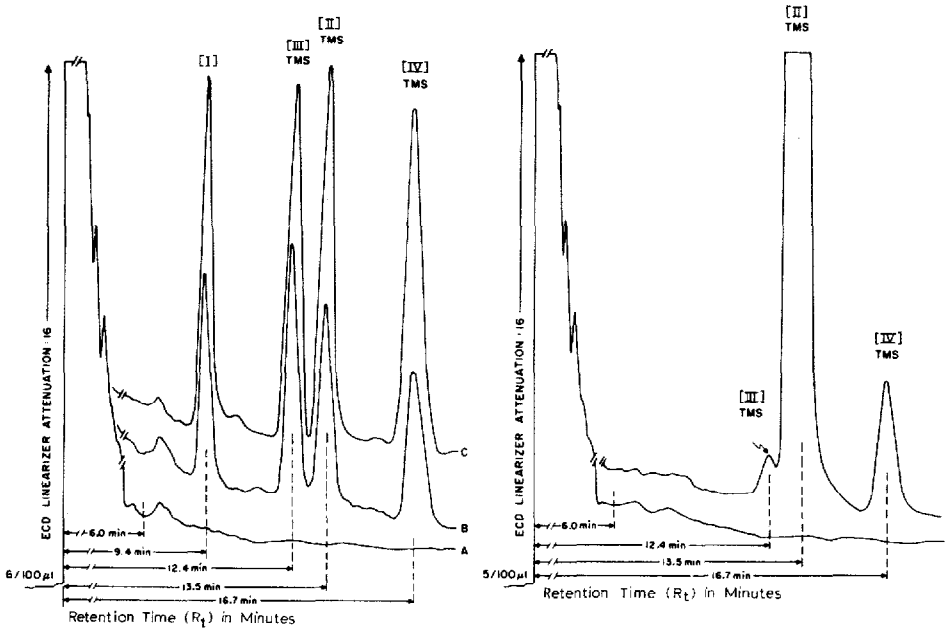


Fig. 9. Chromatograms of the EC—GLC analysis of human urine extracts for midazolam, an imidazo-1,4-benzodiazepine, and its major metabolite. A = control (0 h); B = control urine with added authentic standards; C = authentic standards; D = directly extractable fraction (0—12 h) post dose; E = extractable fraction post glusulase incubation in 0—12 h pooled voidance.

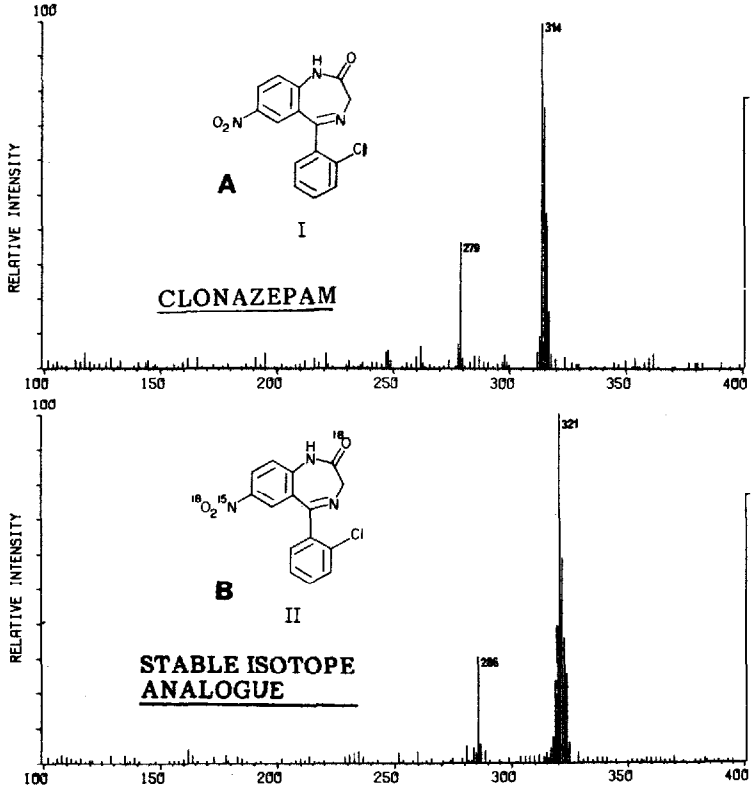
fluorobenzyl bromide or benzoyl chloride which can be quantitated with picogram (10^{-12} g) sensitivity [19].

Analysis by HPLC

HPLC is a most effective means of determining solvent extractable drugs with a minimum of clean-up, if any. Usually, the residue of the sample extract is dissolved in the mobile phase itself, and an aliquot injected directly for analysis using either normal phase or reversed-phase chromatography.

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. Such compounds can be analyzed by the direct injection of an aliquot of the biological sample. 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. Urine is filtered to remove salts and colloidal materials, extracted with diethyl ether or *n*-hexane as a clean-up step (if needed), 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 postcolumn derivatization with fluorescamine [20] (Fig. 11).



	[MH]$^+$	[M-H]$^-$
m/z	316	314

Sensitivity Limit:

Clonazepam $\left\langle \begin{array}{l} [MH]^+ = 1 \text{ ng/ml of plasma} \\ [M\text{-}H]^- = 100 \text{ pg/ml of plasma} \end{array} \right.$

Fig. 10. Methane GC—CI—MS analysis of clonazepam, a 7-nitro-1,4-benzodiazepin-2-one using positive ion (PI) and negative ion (NI) modes.

Analysis in the fluorescence mode (excitation 385, 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.

The clinical utility of the assay was demonstrated in the determination of the urinary excretion profile of amoxicillin and its benzyl-penicilloic acid metabolite (inactive), in a human following the oral administration of a single 250-mg dose. Virtually, quantitative recovery of the dose was achieved in a

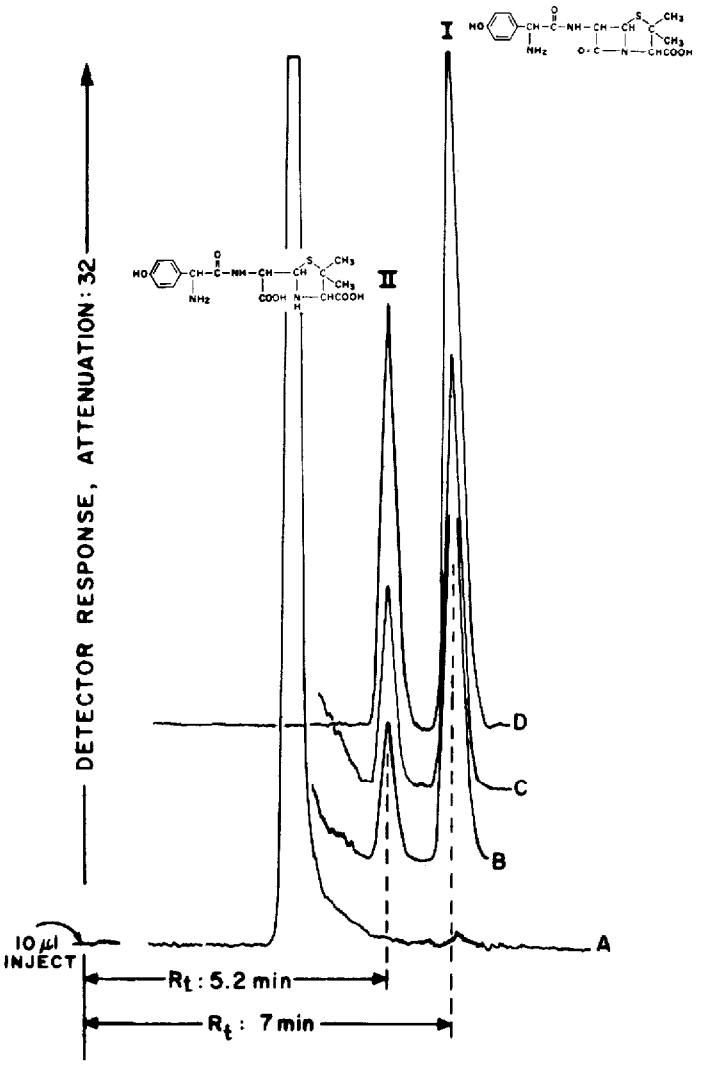


Fig. 11. Chromatograms of the HPLC analysis of amoxicillin [I] and its benzylpenicilloic acid [II] in human urine by fluorometric detection following postcolumn derivatization with fluorescamine. A = diluted control urine; B = post oral dose (250 mg); C = control urine with added authentic standards; D = authentic standards.

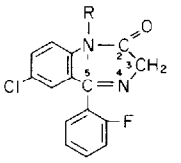
12-h pooled urine collection, making the method a valid non-invasive means of assessing the bioavailability of oral dosage forms [20].

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) [21] 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 enzymatic hydrolysis of a major (key) metabolite in urine and its specific quantitation as the aglycone.

Flurazepam dihydrochloride, [I] · 2HCl, a hypnotic of the 1,4-benzodiazepine class, undergoes extensive biotransformation in man (Fig. 12). The major urinary metabolite N_1-hydroxyethyl-flurazepam [II]-glucuronide accounts for 30–55% of an orally administered dose in a 72-h excretion period (>25% in a 24-h period).



	R-Group		HPLC (RR_t) N.P.	R.P.
FLURAZEPAM	$-(CH_2)_2-N-(C_2H_5)_2$	[I]	0.88	0.96
Mono-desethyl-flurazepam	$-(CH_2)_2-NH-C_2H_5$	[I-A]	0.55	0.75
Di-desethyl-flurazepam	$-(CH_2)_2-NH_2$	[I-B]	2.43	0.65
N-1-hydroxyethyl-flurazepam	$-CH_2-CH_2OH$	[II]	1.00	1.00
N-1-desalkyl-flurazepam	$-H$	[III]	0.89	1.14
N-1-desalkyl-3-hydroxy-flurazepam	$-H$	[IV], 3)CHOH	3.75	0.81
INTERNAL STANDARD for HPLC	$-(CH_2)_2-N-(CH_3)_2$	[V] 2'-Cl	1.56	1.88 (Diazepam)
[II] - glucuronide			-	0.45

Fig. 12. Chemical structures and relative retention times (RR_t) of flurazepam and its metabolites determined by normal phase (N.P.) and reversed-phase (R.P.) HPLC analysis.

Initial studies using reversed-phase HPLC showed the presence of intact [II]-glucuronide in urine directly injected. Upon enzymatic incubation over discrete time intervals ranging from 15 to 150 min followed by direct injection of the incubate (the enzyme was instantly inactivated by the addition of 500 μl of methanol), it was shown that with the progressive diminution of the [II]-glucuronide peak (t_R = 15.0 min), the peak for deconjugated [II] (free base) (t_R = 28 min), increased in height enabling the direct analysis of the hydrolysis of the glucuronide to yield the aglycone [II] which is quantitated against an authentic standard [22].

The tandem detection of the column effluent first by UV at 254 nm followed by differential pulse amperometry (DPA) ensured specificity for the components of interest. The chromatogram monitored by DPA (Fig. 13A) shows two extra components (probably minor metabolites), which are otherwise masked in the solvent front of the chromatogram monitored by UV at 254 nm (Fig. 13B) 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.

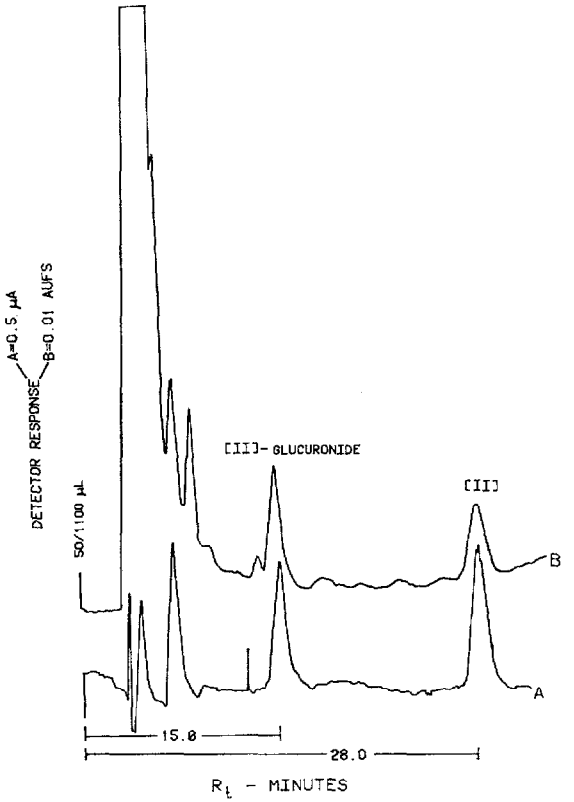


Fig. 13. Chromatograms of reversed-phase HPLC analysis of the metabolite, N_1-hydroxyethylflurazepam [II] in 1—2 h post-dose urine incubated for 30 min, monitored by tandem detection by (A) differential pulse amperometry (DPA) and (B) by UV at 254 nm. Column, μBondapak C_{18}; mobile phase, 0.0075 *M* acetate buffer (pH 3.5)—methanol (1:1); flow-rate, 0.9 ml/min at 1500 p.s.i.

The enzymatic hydrolysis kinetics of [II]-glucuronide yielding [II] (free base) are shown in Fig. 14 and show the progressive diminution of the peak for [II]-glucuronide and the increase in the peak height for [II] (free base) with time. The formation of [II] appeared to plateau after 180 min (3 h) with no significant increment after overnight incubation (16 h) which was then used for expediency in sample throughout.

The urinary excretion profile of [II] was determined by normal-phase (after extraction), and reversed-phase HPLC analysis in a volunteer following the administration of a single 30-mg oral dose of [I] · 2HCl (Dalmane®) (Fig. 15). The total amount of [II] excreted in the 0—48 h interval represented about 10.4 mg of flurazepam · 2HCl (parent drug) equivalent to 35% of the administered dose, demonstrating the feasibility of this approach as a facile non-invasive means of determining pharmacokinetic parameters of a drug [22].

Analysis by thin-layer chromatography

TLC analysis enables rapid development of analytical chromatographic parameters for eventual use in HPLC analysis. Preliminary separation of drug/

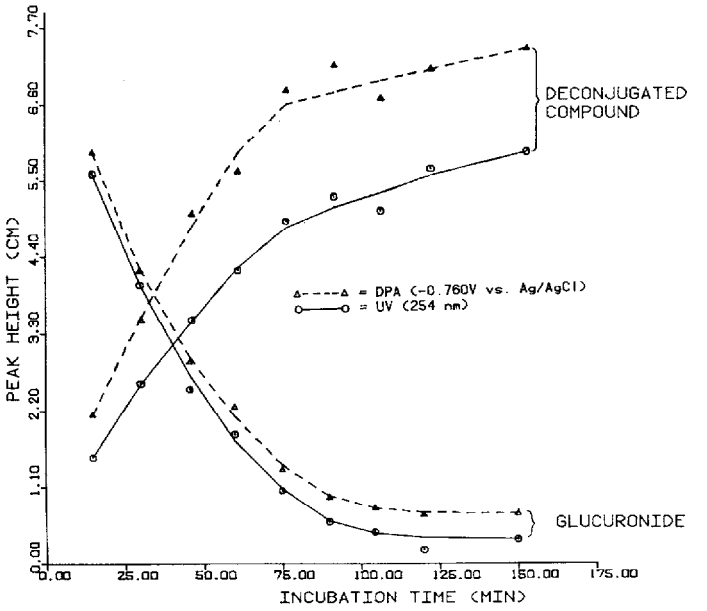


Fig. 14. Kinetics of the enzymatic hydrolysis in urine of [II]-glucuronide to yield N_1-hydroxyethylflurazepam [II] (free base).

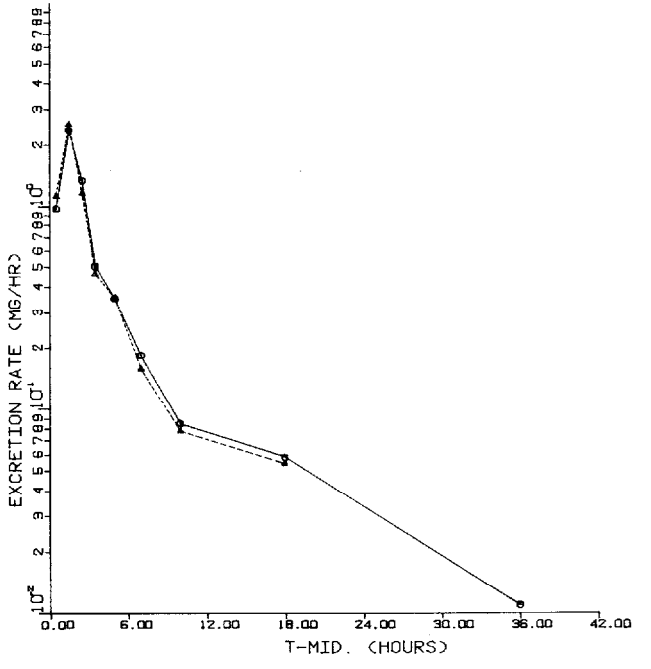


Fig. 15. Urinary excretion profile of hydroxyethylflurazepam [II] determined by normal-phase (Δ - - - Δ) and reversed-phase (o —— o) HPLC analysis.

metabolites using 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. The separated compounds can be eluted from the silica gel and analyzed by a variety of selective techniques, e.g., spectrophotometry/fluorometry, polarography, GLC, GC—MS, nuclear magnetic resonance (NMR), not only for quantitation but also for structure elucidation.

Quantitative analysis by in situ spectrophotometry/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. The sensitivity and specificity of the technique are especially useful in the fluorescence mode and were used in the analysis of flurazepam and its major metabolites in plasma (Fig. 16). The compounds were first hydrolyzed to their *o*-aminobenzophenones which were then cyclized in base (dimethylformamide—potassium carbonate) to the highly fluorescent 9-acridanone derivatives. These were extracted, separated by TLC and quantitated by in situ spectrofluorodensitometry (Fig. 17), and applied to the determination of plasma concentrations of flurazepam and its major metabolites: hydroxyethyl-[II] and N-desalkyl[III], following a single 30-mg oral dose (Fig. 18) [23].

Some of the advantages of TLC analysis include the ability of analyzing the sample by either one- or two-dimensional solvent ascending 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.

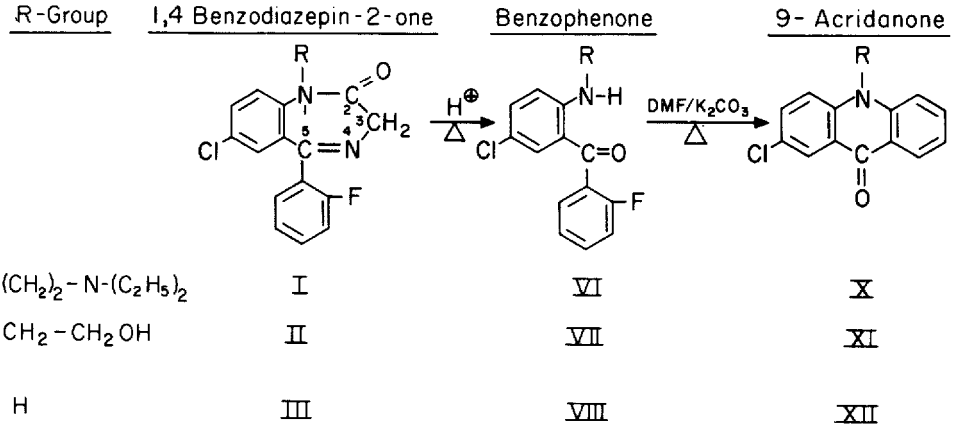


Fig. 16. Chemical reactions of N-1-alkyl-substituted 2'-fluoro-1,4-benzodiazepin-2-ones.

NON-CHROMATOGRAPHIC (DIRECT) ANALYTICAL TECHNIQUES

Absorptiometric and luminescence methods

Spectrophotometric (UV—Vis) and luminescence emission (fluorescence and phosphorescence) analysis [24] have been 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

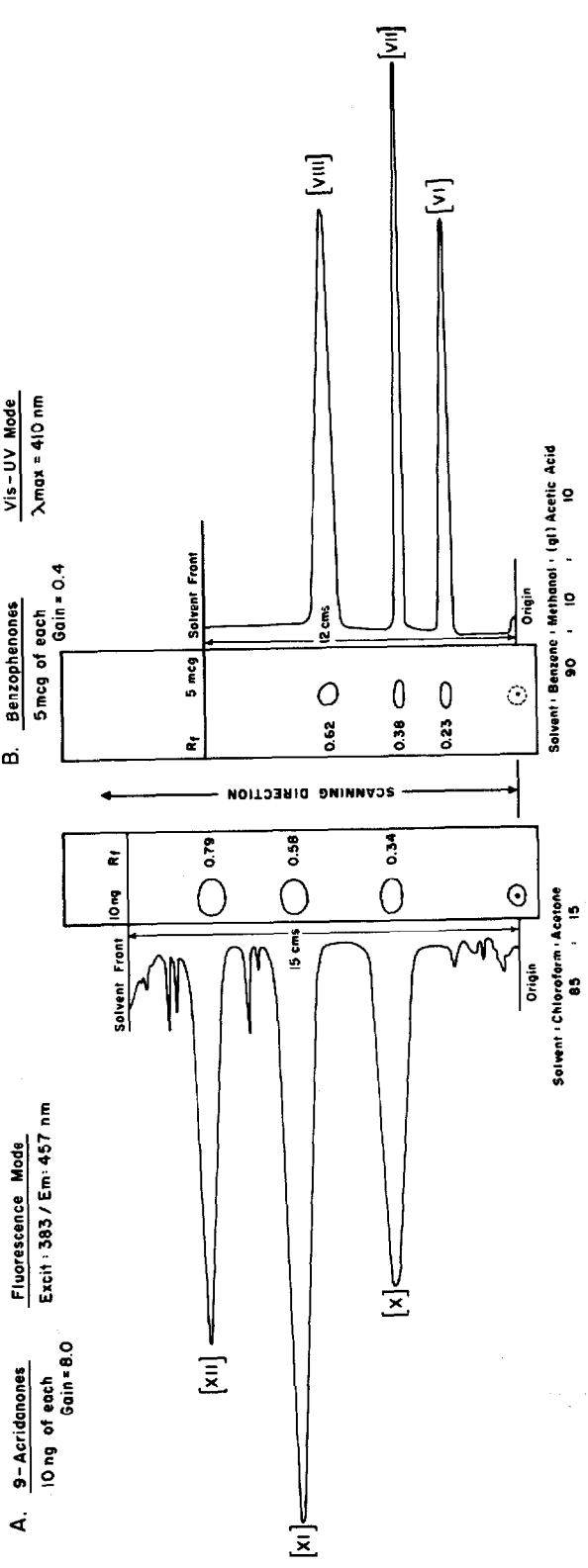


Fig. 17. Thin-layer chromatograms and densitometric scans of (A) the 9-acridanones determined in the fluorescence mode and (B) the benzophenones determined in the Vis—UV mode.

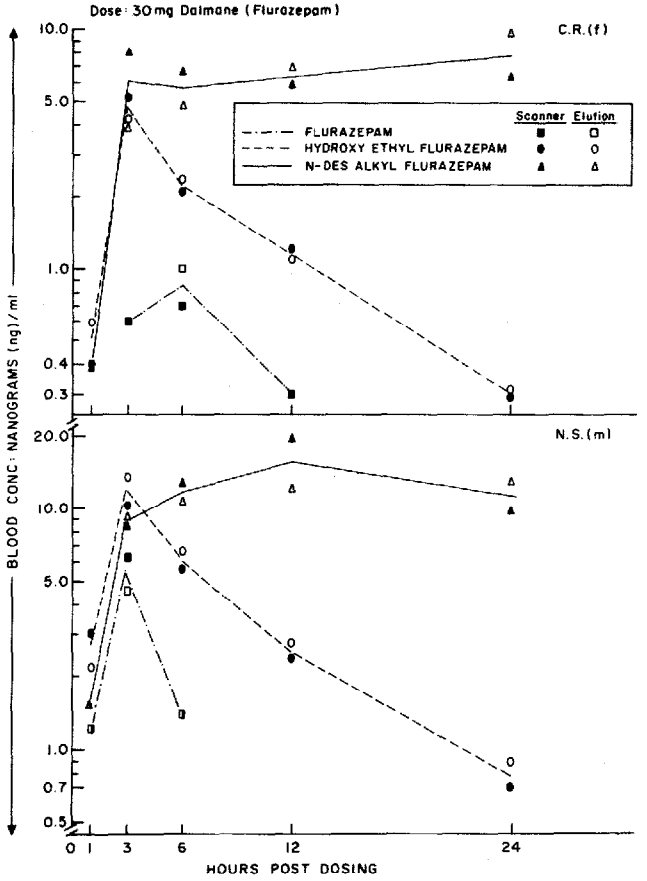


Fig. 18. Blood levels of flurazepam and its major blood metabolites in man determined by fluorometry after elution vs. scanning fluorodensitometry.

present unless used in conjunction with either differential (selective) extraction techniques [1] and/or a chromatographic separation step, e.g. column chromatography, TLC or HPLC.

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]. The DPP analysis of bromazepam (an antianxiolytic agent) and its major metabolites in urine, viz., 3-hydroxybromazepam [II] and 2-amino-3-hydroxy-5-bromobenzoylpyridine [V] which are present mainly as glucuronide/sulfate conjugates, with small amounts of the intact drug [I] and 2-amino-5-bromobenzoylpyridine [IV] is based on selective extraction of the unconjugated from the conjugated fractions prior to polarographic analysis.

The residues of the respective extracts were analyzed directly by DPP in 1.0

M phosphate buffer (pH 5.5) 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 (Fig. 19).

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. The sensitivity was in the order of 50—100 ng of compound per ml of urine analyzed. The recovery of single 12-mg oral doses in adults (equivalent to 0.12—0.18 mg/kg) in a 72-h excretion period ranged from 58– 77% of the administered doses mainly as the glucuronide conjugates of the hydroxylated metabolites.

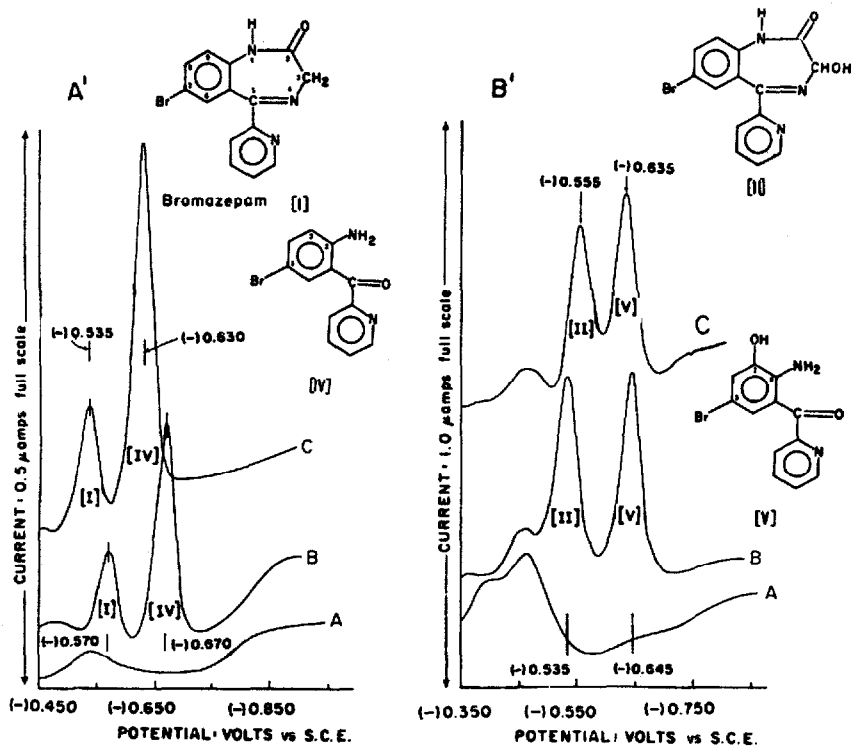


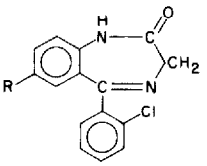
Fig. 19 Differential pulse polarograms of (A') bromazepam [I] and the aminobromobenzylpyridine metabolite [IV] and (B') the 3-hydroxy metabolites [II] and [V] obtained in 1.0 *M* phosphate buffer (pH 5.5) as the supporting electrolyte. (A) Control urine blank, (B) authentic standard mixture, (C) authentic compounds recovered from urine.

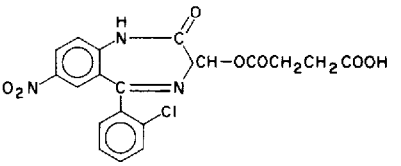
Radioimmunoassay

RIA is a most useful clinical diagnostic tool, especially in monitoring drugs in pediatric therapy where small sample 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. Radioimmunoassays for benzodiazepines [27], e.g. clonazepam, are particularly useful in monitoring pediatric patients on

anticonvulsant therapy which usually involves multiple drug regimens and the need for small (microliter) sampling techniques.

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 Fig. 20.





HAPTEN

R

NO_2 = Clonazepam

NH_2 = 7-Amino-clonazepam

CH_3CONH = 7-Acetylamino-clonazepam

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

Fig. 20. Chemical structure of a specific hapten synthesized for the radioimmunoassay of clonazepam.

The RIA has high sensitivity equal to that of EC—GLC and is in the range of 0.5—1.0 ng of drug per ml of plasma; it can be performed on 100 μl or less of plasma either directly in it or following a simple extraction step with an organic solvent. The excellent correlation between the clonazepam RIA and EC—GLC methods obtained from patients receiving clonazepam alone, and concomitant anticonvulsants attest further to the specificity of the two assays and is shown in Fig. 21.

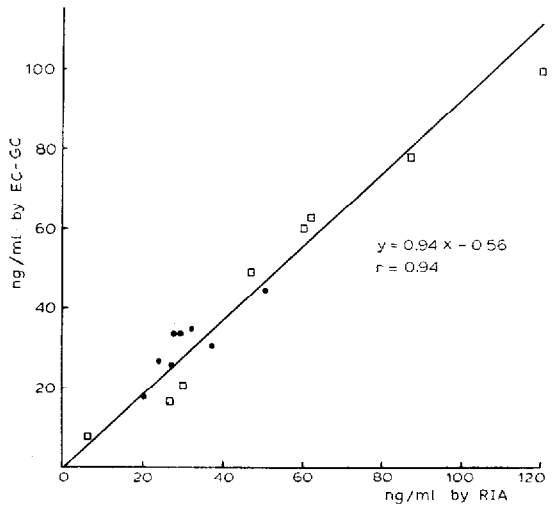


Fig. 21. Correlation between plasma clonazepam concentration determined by RIA and EC—GC in a series of patients receiving clonazepam alone and concomitant anticonvulsants.

CONCLUSION

The chemical structure and the pharmacokinetics of a compound govern 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 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 [28].

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