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Review

Chromatography of benzodiazepines

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CONTENTS

1. INTRODUCTION

Benzodiazepine drugs have hypnotic, tranquillizing and anticonvulsant properties and are frequently encountered in clinical and forensic science casework. Because the world-wide market for benzodiazepine anxiolytics and hypnotics is extremely large, benzodiazepines continue to be developed, evaluated and introduced for clinical use. The assay methods used to determine their concentrations are important to evaluate their pharmacokinetics, bioavailability and clinical pharmacology, and to detect and identify them in toxicological and forensic samples involving road traffic offences and/or drug overdoses. These assay methods include mainly gas chromatography (GC) with electron-capture detection (ECD), high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, gas chromatography–mass spectrometry (GC–MS), radioassay and radioreceptor assay.

This review concentrates on chromatographic techniques. General methods

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dealing with screening of benzodiazepines are cited first, followed by the specific chromatographic assays of individual benzodiazepines.

2 GENERAL ASSAY METHODS

General analytical strategies [1] and methods [2–8] for benzodiazepine monitoring in therapy and toxicology have been published

Pharmacokinetic data compilations on benzodiazepine compounds are available [9–12]. Some of these compilations include assay methods [10,12]. Plasma protein binding and ionization constants have also been described [9]. The pK_{a1} varies between 1 6 (temazepam) and 4 7 (chlordiazepoxide) [9].

Chromatographic, spectroscopic and MS properties of 33 benzodiazepines have been published [13]. The GC retention index values have been determined using a non-polar SE-30-type stationary phase because this is the preferred phase for the screening and identification of drugs [13].

UV spectroscopic data (including maximum wavelength of absorption and specific absorbance) and MS data have also been given. Because many benzodiazepines are readily hydrolysed to give the corresponding aminobenzophenone (Fig. 1) under the conditions used to hydrolyse tissues prior to toxicological analysis, several aminobenzophenones have been prepared from the parent benzodiazepines and their GC, thin-layer chromatographic (TLC) and MS properties have also been described [13].

2.1. Thin-layer chromatography

TLC $R_F \times 100$ values of parent benzodiazepines have been measured on three TLC systems [13]. Two-dimensional TLC identification of twelve 1,4-benzodiazepines or their hydrolysis products, *i.e.* the corresponding aminobenzophenones, has been reported [14]. Methods for the rapid, reliable and sensitive detection and

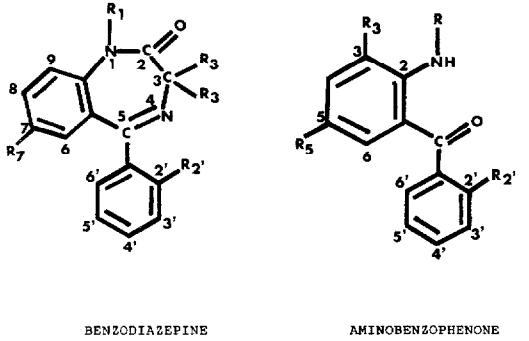


Fig 1 Ring nomenclature for 1,4-benzodiazepines and aminobenzophenones

identification of eighteen 1,4-benzodiazepine drugs (bromazepam, camazepam, chlordiazepoxide, clonazepam, clorazepate, diazepam, flunitrazepam, flurazepam, halazepam, ketazolam, lorazepam, lormetazepam, medazepam, nitrazepam, oxazepam, oxazolam, prazepam and temazepam) have been presented by Schütz [15]. The methods were based on the hydrolysis of the 1,4-benzodiazepines in acidic media to the corresponding aminobenzophenones and their TLC separation using two mobile phases and detection with Bratton–Marshall reagent directly or after photochemical dealkylation. TLC of radioactive benzodiazepine [16] and TLC–densitometric analysis of benzodiazepines [1,17] were also described.

2.2. Detection in other than planar techniques

Liquid–liquid extraction procedures followed by HPLC are not suitable for most of the benzodiazepines, because currently available detectors are insufficiently sensitive and selective [7]. Most of the published work on the liquid chromatography of the benzodiazepines has employed UV absorbance for detection; but nearly all of benzodiazepines are electrochemically reducible at practicable electrode potentials [6]. Despite this, only those compounds carrying very readily reduced substituents show upon electrochemical detection sensitivities competitive with those observed with UV absorbance, mainly because of the interference caused by the reduction of traces of oxygen dissolved in the eluents. To a lesser extent, difficulties have arisen from contamination and irreproducibility of some working electrodes, and from the noise levels generated by dropping mercury electrodes. However, none of these problems remains of significance, and reductive-mode detection employing a pendent mercury drop electrode is now an applied technique [6].

Polarographic methods have been used with success for the determination of the urinary excretion of metabolites of the 1,4-benzodiazepines, owing mainly to the easy reduction of the azomethine group in position 4,5 common to these compounds. Nitro group-containing 1,4-benzodiazepines, *e g.* nitrazepam, clonazepam and flunitrazepam, can likewise be analysed 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 [1].

The most sensitive and specific routine analytical technique at present available is GC with ECD In principle, this method is capable of detecting picogram amount of most benzodiazepines. However, the potential sensitivity of the method has not been achieved owing to absorption and catalytic decomposition of the more polar benzodiazepines on the packed columns that have been used almost exclusively with this technique [7]. Many methods have been introduced to overcome the adsorption problem, such as priming, derivative formation, acid hydrolysis of benzodiazepines to aminobenzophenones and the use of glass SCOT columns in combination with a solid's injector [7]. These methods are useful for

pharmacokinetic studies or the rapid identification of individual benzodiazepines taken in overdose amounts, but they lack the necessary sensitivity and resolution for forensic work.

To overcome the lack of sensitivity and resolution, the use of deactivated Amberlite XAD-7 porous polymer beads to remove interfering co-extractives was proposed, followed by silica capillary column GC–ECD [7]. The Amberlite XAD-7 clean-up improves the ECD selectivity and protects the column and detector from contamination, enabling single-dose therapeutic levels of a wide range of benzodiazepines and metabolites in 0.2-ml blood samples to be analysed.

With GC and HPLC, unknown compounds can be identified only by their retention times thus causing identification problems for compounds with identical retention times. Therefore, combined GC–MS, which allows the identification of a compound by its characteristic ion(s), is nowadays an essential tool. Moreover, the benzodiazepines lend themselves excellently to analysis by GC with chemical ionization MS [1].

2.3. High-performance liquid chromatography

Retention data using both silica and ODS-silica in HPLC have been given [13]. HPLC retention characteristics of 21 benzodiazepines and some of their metabolites have been examined on both silica and ODS-silica (Fig. 2) packing materials [18]. Four HPLC systems have been considered and retention data were presented for the compounds on these four HPLC systems. The correlation of retention data on the systems was considered with reference to the problem of identifying unknown benzodiazepines. An HPLC support was prepared based on silica beads coated with a β-cyclodextrin-containing polymer, which allows the elution of solutes in order of their affinity of β-cyclodextrin [19]. Binding constants were determined from the retention data for drugs (including diazepam) eluted with pure buffer in the new support.

The enantiomers of 46 3-substituted 5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-ones have been chromatographically separated on chiral stationary phases derived from (*R*)-phenylglycine or (*S*)-leucine. Both analytical- and preparative-scale separations have been effected [20] The computer-aided optimization of the resolution of the enantiomers of three closely related benzodiazepines (temazepam, oxazepam and lorazepam) has been attempted on three new column systems: cellulose triacetate, β-cyclodextrin and the reversed-phase column porous graphitic carbon with β-cyclodextrin as a mobile phase additive [21].

Benzodiazepines were extracted with a C_2 AASP cartridge within 5 min. The recovery ranged from 92 to 104% and was independent of the concentration. Using a suitable gradient elution, complete reversed-phase HPLC separation of nineteen benzodiazepines was achieved in 50 min with detection limits of less than 3 ng/ml in urine and 5 ng/ml in other biological fluids. Using a rapid scanning multi-channel detector, the identities of the benzodiazepines can be confirmed [2]

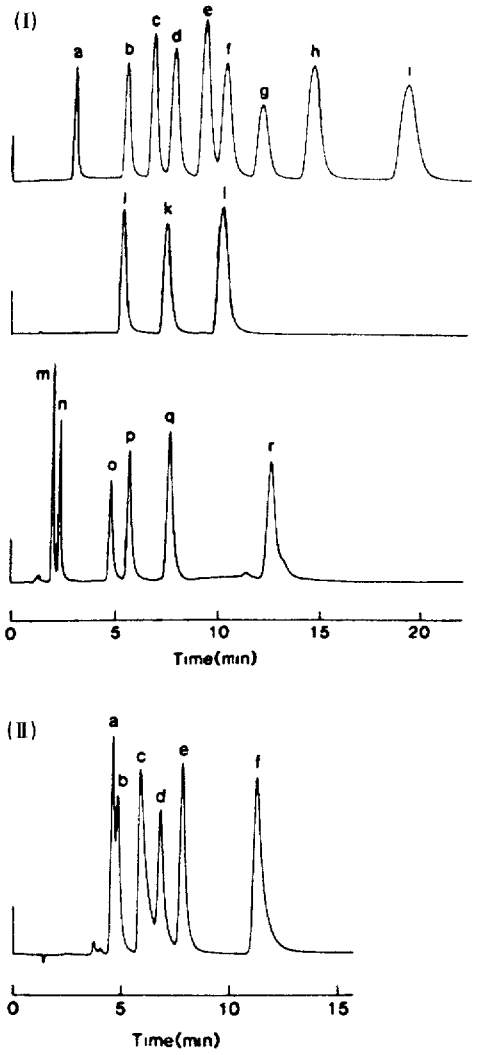


Fig 2 (I) HPLC profiles of benzodiazepines and metabolites on an ODS-silica column (Hypersil, 5 μm, 200 × 5 mm I D), eluent, 55% methanol containing phosphate buffer (pH 7 25), flow-rate, 1 5 ml/min; detection, 240 nm Peaks: a = clorazepic acid, b = nitrazepam; c = clobazam; d = oxazepam, e = temazepam, f = chlordiazepoxide, g = nordazepam, h = diazepam; i = ketazolam, j = clonazepam, k = triazolam; l = lormetazepam; m = 7-aminonitrazepam, n = 7-acetamidonitrazepam; o = demoxepam, p = desmethylclobazam, q = desmethylchlordiazepoxide (from ref. 18 with permission) (II) HPLC profile of benzodiazepines and metabolites on an ODS-silica column (Hypersil, 5 μm, 200 × 5 mm I D); eluent, 70% methanol containing phosphate buffer (pH 7.67); flow-rate, 1 5 ml/min, detection, 240 nm. Peaks· a = diazepam, b = ketazolam; c = flurazepam, d = desoxychlordiazepoxide, e = prazepam, f = medazepam (from ref 18 with permission)

A system has been described that identifies a number of unknown drugs (benzodiazepines, antidepressants and neuroleptics) in blood specimens for clinical–toxicological purposes. Ion-pair HPLC with a photodiode array detector saves the UV spectrum of every chromatographically significant peak. Post-run data processing provided by a microcomputer retrieves candidate substances from a library of UV spectra. Selected standard UV spectra are compared with the unknown by five different similarity tests. The discriminatory efficiency of these algorithms has been determined. Multi-component analysis, a built-in program of the spectrophotometer, provided the most reliable results [3].

Diazepam in serum and phenobarbital in urine have been determined by injecting the untreated sample onto a hydrophobic pre-column, using micellar sodium dodecyl sulphate in the case of serum or phosphate buffer in the case of urine, as the load mobile phase. This traps the components of interest, which are then backflushed onto a microbore analytical column using a stronger mobile phase. Recovery was linear and quantitative over the range 30–3000 ng/ml for diazepam in serum and 2–200 μg/ml for phenobarbital in urine The diazepam method was specific against caffeine and the three major metabolites of diazepam: oxazepam, temazepam and nordiazepam. The effects of varying the pre-column dimensions, pre-column loading time and sodium dodecyl sulphate concentration volume were evaluated [4].

A molecular rearrangement of aminobenzophenones (hydrolysis products of 1,4-benzodiazepines) to 9-acridones has been studied. The compounds synthesized were analysed by HPLC with fluorescence detection, because of their high luminescence. The method can detect sixteen benzodiazepines simultaneously, but ten different aminobenzophenones are formed from the sixteen benzodiazepines considered. It is reliable for the analysis of these compounds in biological samples at therapeutic concentrations [5].

Benzodiazepines in blood samples typical of forensic science work were recovered from 100–250 μl of blood (diluted with aqueous sodium octyl sulphate to suppress protein binding) onto micro-columns of Porapak-T and finally eluted into 60 μl of aqueous acetonitrile. The eluates were taken directly for analysis by HPLC with reductive electrochemical detection at a pendent mercury drop electrode [6].

A single, rapid and specific solid-phase clean-up procedure was developed for the analysis of benzodiazepines and tricyclic antidepressants using a carefully selected wash step and specific sequential elution. Benzodiazepines were eluted from the solid-phase column using water–methanol–acetonitrile (2:3:3) followed by the elution of tricyclic antidepressants with methanol containing 0.6% diethylamine [22]. The separation, isolation and identification of 1,4-benzodiazepine glucuronides (oxazepam, cinolazepam) from biological fluids by reversed-phase HPLC has been reported [23], as has an HPLC method for the determination of triazolobenzodiazepines [24].

The transfer of HPLC methods between laboratories has often been hindered

by the lack of any column standardization. It has long been recognized that packing materials of the same type (*e.g.*, silica, ODS-silica) but from different manufacturers can have very different chromatographic properties. Consequently much time is often wasted in the modification of eluents taken from published procedures in order to achieve the appropriate separations on the commercial brand of packing material available in the laboratory. Furthermore, when a laboratory is engaged in many different HPLC assays any attempt to stock a comprehensive range of commercial products is impractical and prohibitively expensive. Further work should involve the development of HPLC eluents to use with the standardized packing materials suitable for use in drug analysis

It is important for the analyst to adapt a valuably standardized published HPLC method rather than to try to spend time to realize some modifications and to publish that modified version of the method.

2.4. Gas chromatography

A general method for the trace analysis of benzodiazepines and their major metabolites in 0.2-ml blood samples has been described [7]. The method involves solvent extraction of blood with toluene, isolation of the analytes using deactivated Amberlite XAD-7 porous polymer beads, and analysis of the cleaned-up extracts by capillary column GC with ECD. The clean-up technique eliminates lipids and other interfering material, enabling routine analysis of blood extracts to be carried out with no significant deterioration in column or detector performance over a period of many months. The use of fused-silica capillary columns coated with SE-52 and the correct choice of chromatographic conditions permits benzodiazepines of widely differing volatilities and polarities to be analysed without derivative formation. Data for 26 benzodiazepines and metabolites are presented.

2.5. Gas chromatography–mass spectrometry

A GC–MS method for the identification and/or quantification of diazepam, clobazam, flunitrazepam, triazolam, midazolam, oxazepam and lorazepam and some of their desmethylated and hydroxylated metabolites in plasma has been described [8]. Benzodiazepines were extracted from plasma with butyl acetate at pH9; the hydroxylated compounds were then silylated with N,O-bis (trimethylsilyltrifluoroacetamide). Analysis was performed using a compact mass-selective detector operating in the electron-impact mode. Depending on the concentration, identification was performed either by direct comparison of the observed mass spectrum with reference spectra or by the relative intensities of the most intense and characteristic ions in the selected-ion monitoring (SIM) mode. Quantification was performed in the SIM mode using the most intense ion. The intra-assay precision and accuracy were better than 5–6%; linearity was satisfactory up to

1–2 μg/ml. The detection limit was 1–5 ng/ml for most of the benzodiazepines. This method can be easily used in clinical situations when a safe and rapid response is essential for patient treatment.

A method for the identification and differentiation of the following benzodiazepines and their metabolites in urine after acid hydrolysis and acetylation has been published [25]: bromazepam, camazepam, chlordiazepoxide, clobazam, clonazepam, clorazepate, clotiazepam, cloxazolam, delorazepam, diazepam, ethylloflazepate, flunitrazepam, flurazepam, halazepam, ketazolam, loprazolam, lorazepam, lormetazepam, medazepam, metaclazepam, midazolam, nitrazepam, nordazepam, oxazepam, oxazolam, prazepam, quazepam, temazepam and tetrazepam. The acetylated extract was analysed by computerized GC–MS. An online computer allowed rapid detection using ion chromatography with ions *m/z* 205, 211, 230, 241, 245, 249, 312 and 333. The identity of positive signals in the reconstructed ion chromatogram was confirmed by a comparison of the stored full mass spectra with reference spectra. The ion chromatograms, reference mass spectra and GC retention indices on OV-101 are documented.

GC–MS identification of benzodiazepines has been reported by several other authors [5,13,26–32].

3 INDIVIDUAL BENZODIAZEPINE ASSAYS

Many schemes have been proposed to classify the various benzodiazepines. In pharmacokinetics, a useful framework is the categorization according to the range of elimination half-life (ultrashort, short, intermediate or long). Some authors classify the benzodiazepines as chloro- and nitrobenzodiazepines. These compounds are also classified as:

(1) 1,4-Benzodiazepines: bromazepam, camazepam, chlordiazepoxide, clonazepam, diazepam, chlorodesmethyldiazepam, dipotassium chlorazepate, ethyl loflazepate, flunitrazepam, flurazepam, halazepam, lorazepam, lormetazepam, medazepam, nitrazepam, oxazepam, pinazepam, prazepam and quazepam.

(2) 1,5-Benzodiazepines. clobazam.

(3) Imidazo-1,4-benzodiazepines: climazolam and midazolam.

(4) Triazolobenzodiazepines: adinazolam, alprazolam and triazolam.

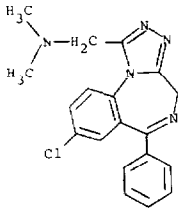
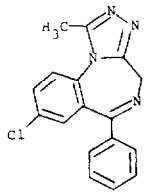
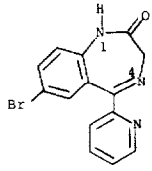
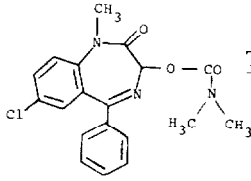
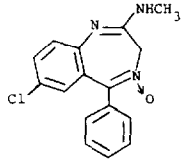
(5) Thienodiazepines: clotiazepam.

Table 1 refers to the monitoring of individual compounds. The benzodiazepines are listed in alphabetical order, with their molecular structures. Most of the referenced chromatographic methods are earlier than 1984. In general, the GC methods used ECD and HPLC methods used UV detectors. Modern HPLC methods used reversed-phase columns, and more and more capillary columns are being used for the GC methods. The chemical reaction of 1,4-benzodiazepines and benzodiazepin-2-ones to yield derivatives that are suitable for GC–ECD analysis were described by De Silva [1].

Chromatographic methods have been applied to plasma, serum, blood, urine

TABLE I

MOLECULAR STRUCTURES, METABOLITES AND SELECTED HPLC, GC AND GC–MS REFERENCES FOR BENZODIAZEPINES

Compound	Structure	Metabolites	References		
			HPLC	GC	GC–MS
Adinazolam		Desmethyl	33,34		
Alprazolam		1-Hydroxymethyl 4-Hydroxy	35		
Bromazepam		3-Hydroxy	36	37	
Camazepam		Temazepam		38,39	
Chlordiazepoxide		Desmethyl Demoxepam N-Desmethyldiazepam	40–43		

(Continued on p 468)

TABLE 1 *(continued)*

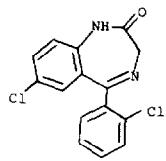
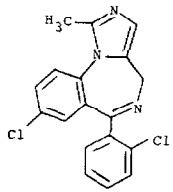
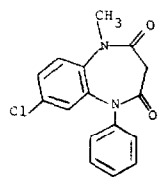
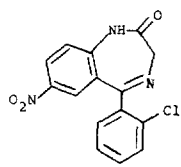
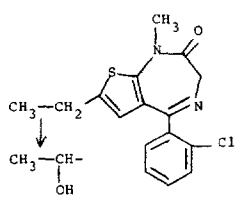
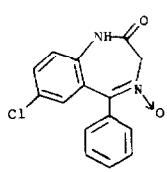
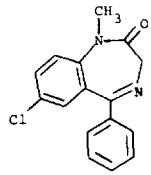
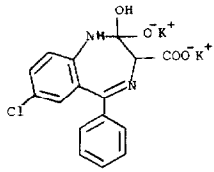
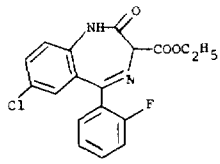
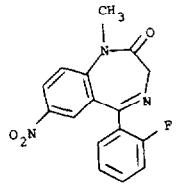
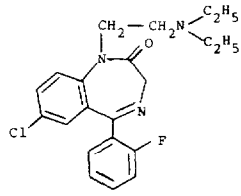
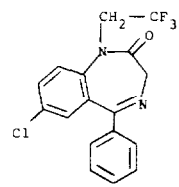
Compound	Structure	Metabolites	References		
			HPLC	GC	GC–MS
Chlorodesmethyl-diazepam		Lorazepam		44	
Climazolam				45	
Clobazam		N-Desmethyl 4′-Hydroxy 4′-Hydroxy-N-desmethyl	46–48	49	
Clonazepam		3-Hydroxy 7-NH_2 7-Acetamido	48,50–52	52–57	
Clotiazepam		N-Desmethyl Hydroxy		58	
Demoxepam		N-Deoxydemoxepam Oxazepam	^{14}C-TLC		59

TABLE 1 *(continued)*

Compound	Structure	Metabolites	References		
			HPLC	GC	GC–MS
Diazepam		N-Desmethyl (nordiazepam) Oxazepam Temazepam	4,60–66	67–72	26
Dipotassium chlorazepate		N-Desmethyldiazepam (nordiazepam)	73	74	
Ethyl loflazepate		Loflazepate Descarboxyloflazepate 3-Hydroxydecarboxy-loflazepate	16		
Flunitrazepam		N-Desmethyl 7-Amino 3-Hydroxy	75	54,55, 76–78	
Flurazepam		N-Desalkyl N-Hydroxyethyl 3-Hydroxy-N-desalkyl Didesethyl Monodesethyl	79–82	78,83	27
Halazepam		Desmethyldiazepam	84	85	

(Continued on p 470)

TABLE 1 *(continued)*

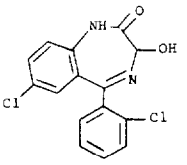
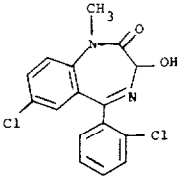
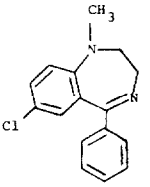
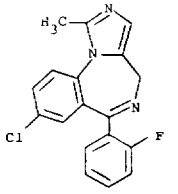
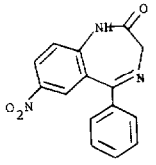
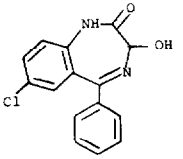
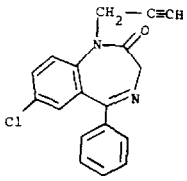
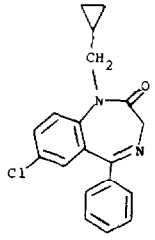
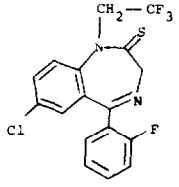
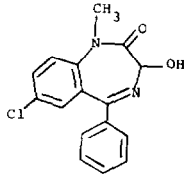
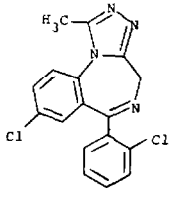
Compound	Structure	Metabolites	References		
			HPLC	GC	GC–MS
Lorazepam		Hydroxy	86	43,87,88	28
Lormetazepam				89	
Medazepam		Diazepam N-Desmethyl Oxazepam		67,90	
Midazolam		1-Hydroxymethyl 4-Hydroxy 1-Hydroxymethyl-4-hydroxy	91–93	94–96	29
Nitrazepam		7-Amino 7-Acetamido 2-Amino-5-nitrobenzophenone ?	48,97,98 TLC and densitometry [17]	57, 99–102	
Oxazepam				103,104	

TABLE 1 *(continued)*

Compound	Structure	Metabolites	References		
			HPLC	GC	GC–MS
Pinazepam		N-Depropargyl 3-Hydroxy Oxazepam	105		
Prazepam		N-Desmethyldiazepam 3-Hydroxy Oxazepam		106	
Quazepam		2-Oxoquazepam N-Destrifluoroethyl		107	
Temazepam			108		
Triazolam		1-Hyroxymethyl 4-Hydroxy	109	30,110	

and tissue samples. A flow diagram of the extraction procedures used for the analysis of various 1,4-benzodiazepines and 1,4-benzodiazepin-2-ones in whole blood has been presented by De Silva [1].

With HPLC methods, the limit of quantitation of the compound varied between 10 and 50 ng/ml; with GC methods, it was between 2 and 20 ng/ml; with GC–MS methods, sensitivities between 100 pg/ml and 1 ng/ml were reached. These methods allow the determination of therapeutic concentrations of benzodiazepines. One example for HPLC, GC and GC–MS is given in Figs. 3–5, respectively. With some benzodiazepines, *e g.* lorazepam [86], and in single-dose pharmacokinetic studies or forensic medicine, low plasma concentrations are attained.

The known metabolites of the different benzodiazepines are listed in Table I and the active ones are underlined. The glucuronides are not listed. The biotransformation of benzodiazepines to metabolites common to several benzodiazepine drugs has been described by De Silva [1]

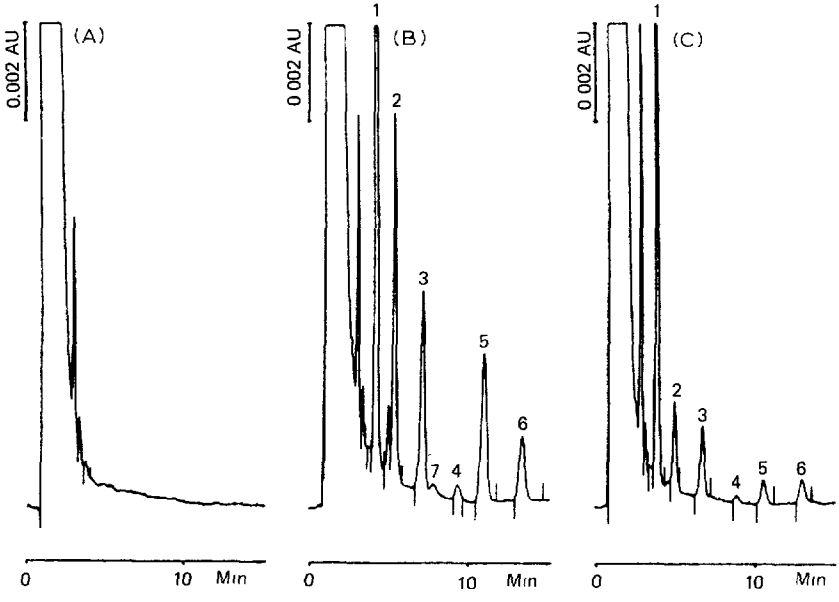


Fig 3 HPLC profiles of (A) drug-free human plasma, (B) plasma taken from a volunteer 1 5 h after oral administration of 30 mg of flurazepam and (C) plasma spiked at concentrations of 1 04, 4 97, 5 60, 4 96 and 5.40 ng/ml of flurazepam, compounds 3, 2, 5 and 6, respectively Chromatographic conditions column, C_8, 12 5 cm × 0 46 cm I.D , mobile phase, 18 5% acetonitrile, 18 5% methanol in 0 1 *M* sodium phosphate monobasic (pH 4 1), flow-rate, 1 5 ml/min, detection wavelength, 230 nm at 0.01 a u.f s Peaks 1 = pronethanol (internal standard), 2 = didesethylflurazepam, 3 = monodesethylflurazepam, 4 = flurazepam, 5 = hydroxyethylflurazepam, 6 = desalkylflurazepam, 7 = unknown Vertical bars represent integration marks (from ref. 82 with permission)

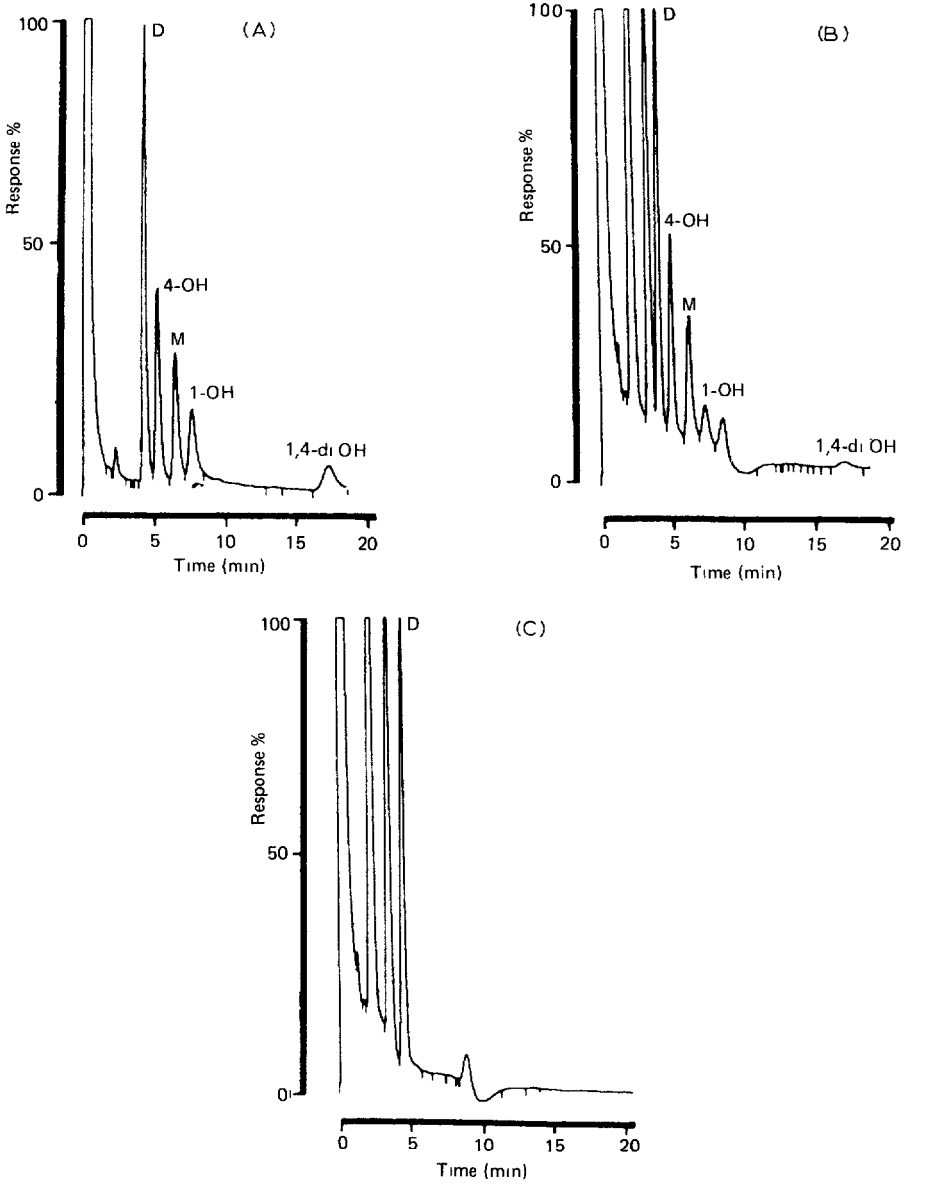


Fig 4. GC–ECD profiles of (A) unextracted standard sample containing 20 ng/ml midazolam and its metabolites, (B) extracted standard sample containing 20 ng/ml midazolam and its metabolites and (C) blank plasma sample Peaks. D = diazepam, 4-OH = 4-hydroxymidazolam, M = midazolam; 1-OH = 1-hydroxymethylmidazolam, 1,4-diOH = 1-hydroxymethyl-4-hydroxymidazolam The column was an HP-17 cross-linked capillary wide-bore column (50% Ph-Me-silicone, 10 m × 0 53 mm I D , 2 0 μm film thickness (from ref 95 with permission)

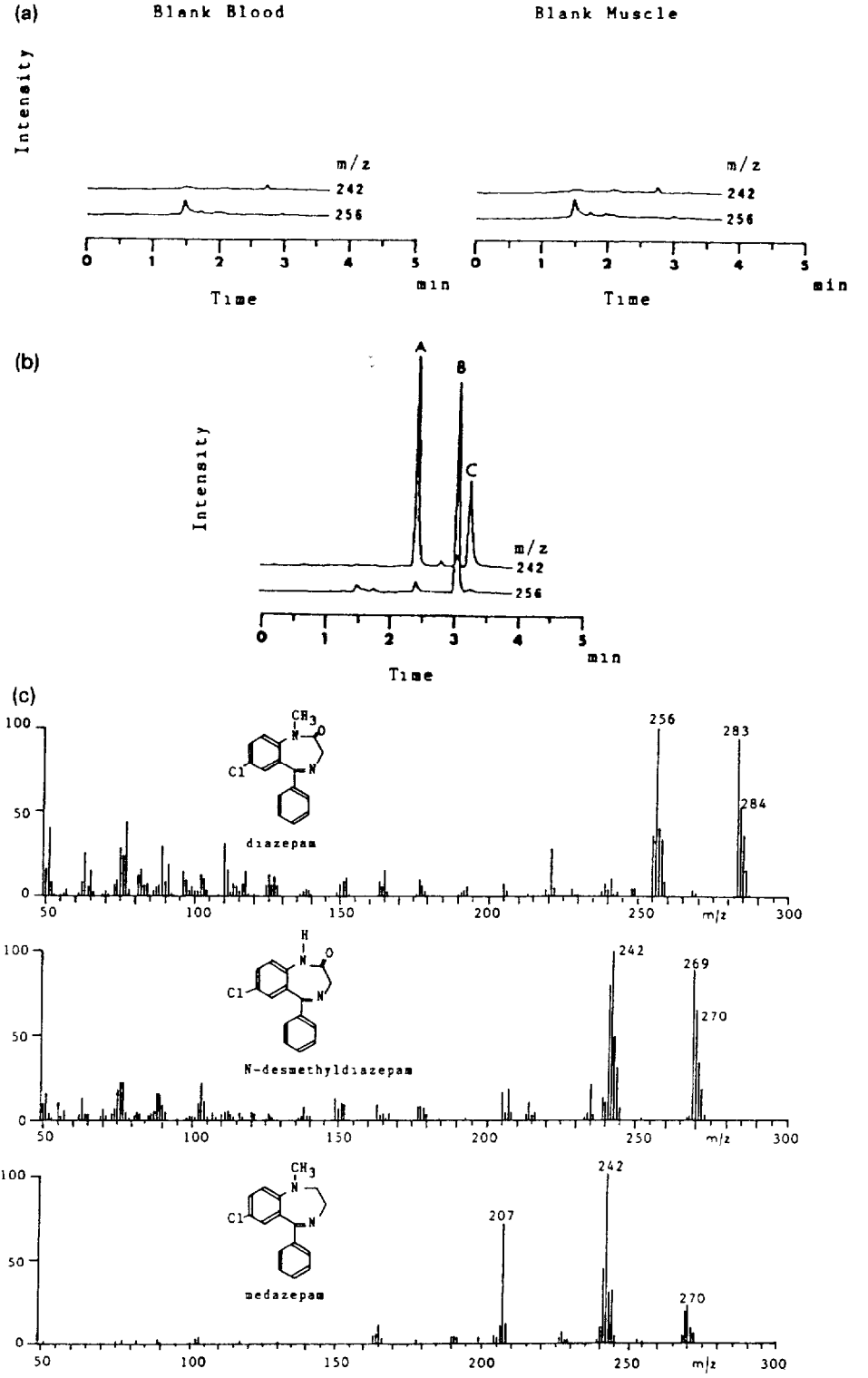


Fig 5 (a) Mass fragmentograms of an extract from drug-free whole blood and skeletal muscle (b) Mass fragmentograms of an extract from blood containing 100 ng/g each of (B) diazepam, (C) N-desmethyldiazepam and (A) I S (medazepam) (c) Mass spectra of diazepam, N-desmethyldiazepam and medazepam (from ref 26 with permission)

4 STABILITY AND SPECIFICITY

It has been reported that nitrazepam can be estimated by GC of the intact molecule, but it elutes as a broad peak, with a sensitivity barely adequate for analysis of the drug levels usually encountered in clinical plasma samples. To increase the sensitivity and improve peak shape, nitrazepam was hydrolysed to 2-amino-5-nitrobenzophenone (ANB) prior to chromatography [99]. It has also been reported that after oral administration, the major urinary metabolites of nitrazepam were 7-aminonitrazepam and 7-acetamidonitrazepam; the parent drug and ANB were excreted in small amounts [17]. Thus the GC method used [99] to determine nitrazepam in urine cannot be specific owing to the presence of ANB (Fig. 1).

The photochemical decomposition and thermal instability of 1,4-benzodiazepines in biological fluids has been reported previously [97,111]. In order to test whether clobazam and its active metabolite were stable under conditions previously shown to cause decomposition of 1,4-benzodiazepines, five plasma and five serum samples collected from patients receiving clobazam on a chronic basis were exposed to light at room temperature for 72 h. When replicate analyses on fresh and stored samples were performed [49] and the resultant data were subjected to statistical analysis, no significant difference in clobazam or N-desmethylclobazam concentrations was found between samples. The presence of the more stable carboxamide group in place of the readily hydrolysed imine group found in the 1,4-benzodiazepines would be an appropriate explanation for these stability differences and would be consistent with the observation that ring-opened molecules have been detected among the metabolites of some 1,4-benzodiazepines, whereas the metabolites of clobazam all retain the diazepine ring.

Some medications are currently associated with clobazam in the treatment of epilepsy. It has been reported [47] that the metabolite N-desmethyldiazepam (nordiazepam) interfered with the analysis of clobazam and N-desmethylclobazam.

A major and early step in the biotransformation of ethyl loflazepate (Table 1) is the hydrolysis of the ester bond, leading to the formation of the anionic derivative loflazepate. Owing to its chemical instability, loflazepate cannot be directly extracted from biological samples and is generally extracted and quantified after its decarboxylation to descarboxyloflazepate. As this compound may also be a metabolite of ethyl loflazepate, the conversion of loflazepate into descarboxyloflazepate should be realized after exhaustive extraction of the latter [16]. GC of lorazepam is hampered by the thermal instability of the 3-hydroxybenzodiazepine ring (Table 1), making GC methods difficult to standardize. The methodology requires the use of another 3-hydroxybenzodiazepine as internal standard and the assumption that thermal molecular rearrangement on-column occurs to the same extent with lorazepam and the internal standard [86].

Although medazepam and diazepam (Table 1) are structurally analogous

compounds, the presence of the methylene group at C-2 in the benzodiazepine ring stabilizes medazepam to acid hydrolysis (6*M* hydrochloric acid), whereas diazepam is quantitatively converted into 2-methylamino-5-chloro-benzophenone (MACB) [67]. Alprazolam can undergo ring-opening under acid conditions [112] (Fig 6). Sodium borate was used in the extraction procedure to adjust the pH in order to maintain the diazepine ring structure [35].

Alprazolam is extensively metabolized: 29 metabolites have been identified in urine [12]. Other benzodiazepines are also metabolized. Caution should be taken as regards the specificity (selectivity) of the analytical methods used to determine benzodiazepines.

Benzodiazepines are stable in biological media when stored at −20°C for several weeks or months. But attention should be given to the time elapsed between sampling, centrifuging (if necessary) and storing at −20°C.

5 VALIDATION OF THE CHROMATOGRAPHIC METHODS

Many authors did not validate their published chromatographic method precisely. Within-day precision and accuracy should be determined when a calibration curve had to be run every working day

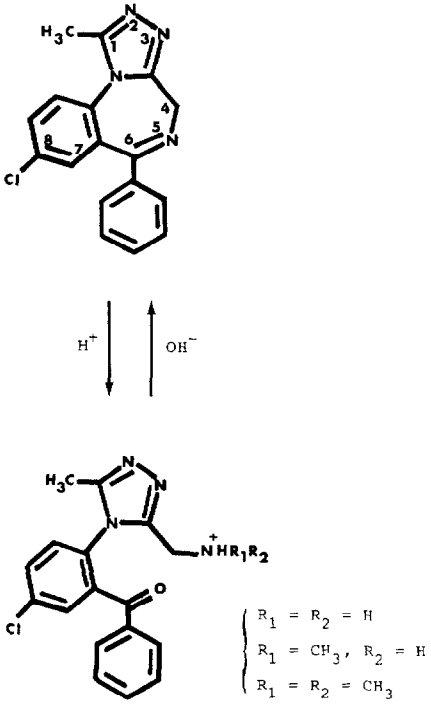


Fig 6 Ring opening of alprazolam [112]

Day-to-day precision and accuracy should be determined when a calibration curve had to be run every week or every two of three days. The following data should be reported to express the validation results:

Concentration added, A.

Mean concentration found, F.

Relative standard deviation, R.S.D. [(S.D./mean) × 100%].

Relative error, $[(F-A)/A] \times 100\%$.

The R.S.D. values should be given for the limit of quantitation. A clear distinction should be made between the limit of quantitation with an R.S.D. of ± 10% and a relative error in the interval 100 ± 5% and the limit of detection.

6 TOXICOLOGICAL SCREENING METHODS

The determination of benzodiazepines in biological fluids requires specific and sensitive techniques that can detect not only the parent drugs but also their metabolites, which are often pharmacologically active.

Although many techniques have become available for the analysis of benzodiazepines and their metabolites in body fluids, difficulties remain in the detection and quantitation of these compounds in the blood samples routinely encountered in forensic casework [6]. Such samples are varyingly and usually extensively haemolysed and are often putrefied. Large amounts of solidified and coagulated materials may be present, and there may be serious contamination by plasticizers and by components of the elastomers, *e.g.* in rubber septa, that the samples may come into contact with during their collection and storage. The amounts of blood available for examination may be small and further restricted by the requirements of other analyses. Radioimmunoassay is currently the only technique that is capable of screening small blood samples [7]. However, it gives no indication of the identity of the benzodiazepine or metabolite detected, and positive results must be confirmed by an alternative method, which commonly depends on chromatography in some form and in turn depends considerably for their effectiveness on an efficient sample preparation procedure [2,3,5–8,26,75,86]. Reversed-phase liquid chromatography in conjunction with microcolumn clean-up techniques should be developed for benzodiazepines.

7 CONCLUSIONS

Methods used to determine benzodiazepines in biological media include GC with ECD, HPLC with UV, GC–MS, radioimmunoassay and radioreceptor assay The available immunoassay for benzodiazepines are unable to discriminate between different benzodiazepines, owing to cross-reactivity of the antibodies used in this type of assay.

The choice of an appropiate assay methods depends on the characteristics of the benzodiazepine, the expertise of the analyst, the equipment available, the

desired sensitivity and specificity and the time involved in method development or adaption and validation

The method used most frequently is the highly sensitive and specific GC–ECD technique. The other methods have limitations. The feasibility of directly assaying drugs in physiological fluids using on-line preconcentration and microbore HPLC should be applied to benzodiazepines more frequently.

8 SUMMARY

An overview of methods for the determination of benzodiazepines in biological media, based on the application of chromatographic techniques, is presented. A general discussion of the techniques in terms of stability, selectivity, validation, standardization, detection and sensitivity is given. No single technique can be claimed as the method of choice for benzodiazepines. Gas chromatography with electron-capture detection has some strong claims and shows generally good sensitivity and reproducibility. High-performance liquid chromatographic equipment is readily available in most laboratories.

The ultimate choice of an assay method for benzodiazepines will be determined by the clinical application (routine monitoring, pharmacokinetics, overdose, forensic medicine) and by the characteristics of the benzodiazepine, the expertise of the analyst, the equipment available, the desired sensitivity and specificity and the time involved in method development or adaptation and validation.

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