ELSEVIER

Journal of Chromatography B, 713 (1998) 201–225

JOURNAL OF CHROMATOGRAPHY B

Review

Methods for the measurement of benzodiazepines in biological samples

Olaf H. Drummer

Department of Forensic Medicine, Monash University, Victorian Institute of Forensic Medicine, 57–83 Kavangh Street, Southbank, Melbourne 3006, Australia

Abstract

A review of methods for the measurement of benzodiazepines in biological specimens published over the last five years is presented. A range of immunoassay procedures using EIA, ELISA, FPIA, agglutination or kinetic interaction of microparticles, or RIA methods are now available. Cross reactivities to benzodiazepines are variable such that no one kit will recognise all benzodiazepines and their relevant metabolites at concentrations likely to be encountered during therapeutic use. Prior hydrolysis of urine to convert glucuronide metabolites to immunoreactive substances improves detection limits for many benzodiazepines. Several radioreceptor assays have now been published and show good sensitivity and specifity to benzodiazepines and offer the advantage (over immunoassay) of being able to detect these drugs with equal sensitivity. Solvent extraction techniques using a variety of solvents were still popular and offer acceptable recoveries and lack of significant interference from other substances. A number of papers describing solid phase extraction procedures were also published. Direct injection of specimens into a HPLC column with back flushing were also successfully described. Seventy two chromatographic methods using HPLC, LC–MS, GC and GC–MS methods were reviewed. HPLC was able to achieve detection limits for many benzodiazepines using UV or DAD detection down to 1–2 ng/ml using 1–2 ml of urine or serum (blood). ECD detectors gave detection limits better than 1 ng/ml from 1 ml of specimen, which was an order of magnitude lower than for NPD. EI-MS offered similar sensitivity, whilst NCI-MS was capable of detection down to 0.1 ng/ml. Methods suitable for the separation of enantiomers of benzodiazepines have been described using HPLC. Electrokinetic micellar chromatography has also been shown to be capable of the analysis of benzodiazepines in urine.

Keywords: Reviews; Benzodiazepines

Contents

0378-4347/98/$19.00
PII: S0378-4347(97)00537-9

1. Introduction

Benzodiazepines are a large class of drugs with some 35 controlled by the United Nations Convention (1971), and many more are available throughout the world [1].

They are commonly used by the community as minor tranquillisers, hypnotics, muscle relaxants and as anticonvulsants. They are often abused by the young illicit drug user often in large doses causing profound behavioural effects. Their continued abuse leads to dependence. The older population are not immune to using benzodiazepines and are also often dependent on their effects [2–6]. Benzodiazepines may also cause or contribute to sudden death if misused [7].

Since benzodiazepines are widely seen in clinical and forensic cases, their measurement in specimens are widely practised [8,9]. Chromatographic techniques, particularly HPLC, GC and GC–MS, are most commonly used to identify specific benzodiazepines present in a specimen initially screened by one of the immunoassay-based kit methods [9,10].

A review of GC–MS methods for the analysis of benzodiazepines was published in 1992 [11,12]. This paper reviews methods published in the scientific literature over the last 5 years for the measurement of benzodiazepines in clinical and post mortem specimens.

2. Methods

2.1. Choice of references

Articles were searched using the Medline database on CD-ROM from January 1992 to April 1997 using >exp*benzodiazepines/bl,ur< as search string. Methods cited from these references or other methods available to the author were also included, where relevant, and covering the time period above.

2.2. Definitions and terms used

Standard abbreviations used by this Journal are used in the review. Abbreviations used are included in the List of Non-Standard Abbreviations (Table 1).

Benzodiazepines are named according to their internationally-accepted nonproprietary names (INN) or their common names. Metabolites are included in the summary tables where applicable.

3. Structural features of benzodiazepines

The classical benzodiazepines are based on the 5-aryl-1,4-benzodiazepine structure, characterised by a benzene ring fused on the 10- and 11-positions of the 1,4-diazepine ring (Fig. 1). The aryl substituent at the 5-position is usually phenyl (e.g., oxazepam) or 2-halophenyl (lorazepam, flurazepam) [13,14].

The 1,4-benzodiazepines include diazepam, nordazepam (also known as nordiazepam), temazepam, oxazepam, which are interconvertible, and are all primarily metabolised and excreted as oxazepam and oxazepam glucuronide. Clorazepate and chlordiazepoxide are also metabolised to oxazepam through nordazepam. Other members such as ketazolam, camazepam, oxazolam, pinazepam, prazepam, halazepam and medazepam are prodrugs

Table 1
List of nonstandard abbreviations

ACN	Acetonitrile	B	Blood
BuCl	1-Butylchloride	BSTFA	*N,O*-bis-(trimethylsilyl)-trifluoroacetamide)
CD-ROM	Compact disc-read only memory	CH	Cyclohexyl
CEDIA	Cloned enzyme donor immunoassay	DMS	Dimethylpolysiloxane
CI	Chemical ionisation	DAD	Diode-array detector
CNPP	Cyanopropylphenyl	d.a.u.	Drugs of abuse
DCM	Dichloromethane	ECD	Electron capture detector
EIA	Enzyme immunoassay	EI	Electron impact (MS)
EMIT	Enzyme multiplied immunoassay	FPIA	Fluorescent polarisation immunoassay
F	Fluorescence	FSC	Fused silica column
GC	Gas chromatography	GC–MS	Gas chromatography–mass spectrometry
FID	Flame ionisation detector	INN	International nonproprietary name
MECC	Micellar electrokinetic chromatography	MeI	Methyl iodide
MeOH	Methanol	MSA	Methane sulfonic acid
MS–MS	Tandem mass spectrometry	MTBSTFA	*N*-methyl-*N*-(*tert.*-butyldimethylsilyl)-trifluoroacetamide
NBZ	Nitrobenzodiazepine	NCI	Negative ion chemical ionisation
NPD	Nitrogen phosphorous detector	OH	Hydroxy
P	Plasma or serum	PI	Positive ion (MS)
PM	Post mortem	TBABr	Tetrabutylammonium bromide
PMS	Diphenyldimethylsiloxane	RIA	Radioimmunoassay
RRA	Radio receptor assay	SDS	Sodium dodecyl sulfate
SPE	Solid phase extraction	SPME	Solid-phase micro extraction
TEAP	Triethyl phosphate	TMAH	Tetramethylammonium hydroxide
TEA	Triethylamine	TFPMS	Trifluoropropylmethylsiloxane
TMCS	Trimethylchlorosilane	U	Urine

which are metabolised to oxazepam through either diazepam, nordazepam or temazepam. Oxazepam is therefore a common target metabolite for most 1,4-benzodiazepines in urine.

The nitrobenzodiazepines, flunitrazepam, clonazepam, nitrazepam and nimetazepam are all substituted 1,4-benzodiazepines with a nitro group at R_7 and have a common pathway to a 7-amino metabolite, which is a target metabolite in urine and in postmortem blood specimens.

Flurazepam and ethyl loflazepate are rapidly dealkylated to the target 1,4-benzodiazepine desalkyl flurazepam. The 2-hydroxyethyl flurazepam is the major urinary metabolite (target metabolite) of flurazepam [15].

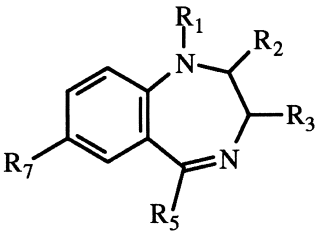
Other variations of the benzodiazepine structure include a 1,3-diazole (imidazole) annulation on the 1,2-position to form imidazo or diazolo benzodiazepines such as midazolam, clinazolam and loprazolam (Fig. 2). The triazolobenzodiazepines alprazolam, estazolam and triazolam have a 1,2,4-triazole ring in place of the diazole ring. Adinazolam is a triazolo benzodiazepine which is metabolised to estazolam and α-hydroxy-alprazolam [16].

Other structural modifications include ring formation on the 4,5-position (e.g., haloxazolam), and replacement of the primary benzene ring with a thienyl ring (brotizolam, clotiazepam and bentazepam), while clobazam is an 1,5-benzodiazepine (Fig. 3).

Many benzodiazepines in acid solution will hydrolyse, forming the corresponding benzophenone. These benzophenones can be targeted if desired [17,18].

Most benzodiazepines are extensively metabolised by phase I (predominantly dealkylation, aliphatic and aromatic hydroxylation, reduction, and acetylation), and phase II (conjugation) reactions. In most cases the phase I metabolites have some biological activity which may be greater or less than that of the parent, whereas the conjugates possess no significant activity. Conjugates consist largely of glucuronides. The metabolism of many benzodiazepines is described elsewhere [10,17–20].

Flumazenil acts as an antagonist and imidazenil acts as a partial agonist on benzodiazepine receptors, these are structurally related to the benzodiazepines themselves (Fig. 3).



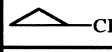
Benzodiazepine	R_1	R_2	R_3	R_5	R_7
Bromazepam	H	=O	H	2'-pyridyl	Br
Camazepam	CH_3	=O	DMACO	Phenyl	Cl
Clonazepam	H	=O	H	2-Cl-phenyl	NO_2
Clorazepate	H	=O	COOH	Phenyl	Cl
Chlordiazepoxide[1]	-	CH_3NH	H	Phenyl	Cl
Delorazepam	H	=O	H	2-Cl-phenyl	Cl
Diazepam	CH_3	=O	H	Phenyl	Cl
Ethyl loflazepate	H	=O	CH_3CH_2COO	2-F-phenyl	Cl
Fludiazepam	CH_3	=O	H	2-F-phenyl	Cl
Flunitrazepam	CH_3	=O	H	2-F-phenyl	NO_2
Flurazepam	DEAE	=O	H	2-F-phenyl	Cl
Halazepam	CF_3CH_2	=O	H	Phenyl	Cl
Lorazepam	H	=O	OH	2-Cl-phenyl	Cl
Lormetazepam	CH_3	=O	OH	2-Cl-phenyl	Cl
Medazepam	CH_3	H	H	Phenyl	Cl
Nordazepam	H	=O	H	Phenyl	Cl
Nimetazepam	CH_3	=O	H	Phenyl	NO_2
Nitrazepam	H	=O	H	Phenyl	NO_2
Oxazepam	H	=O	OH	Phenyl	Cl
Pinazepam	$CH{\equiv}C-CH_2$	=O	H	Phenyl	Cl
Prazepam		=O	H	Phenyl	Cl
Quazepam	CF_3CH_2	=S	H	2-F-phenyl	Cl
Temazepam	CH_3	=O	OH	Phenyl	Cl
Tetrazepam	CH_3	=O	H	1,2-dehyd-rocyclohexyl	Cl

Fig. 1. Structures of selected 1,4-benzodiazepines. [1]N_4=N-oxide and double bond at C_1–C_2, DMACO=*N*-dimethylaminocarbonyloxy–, DEAE=*N*-diethylaminoethylene–.

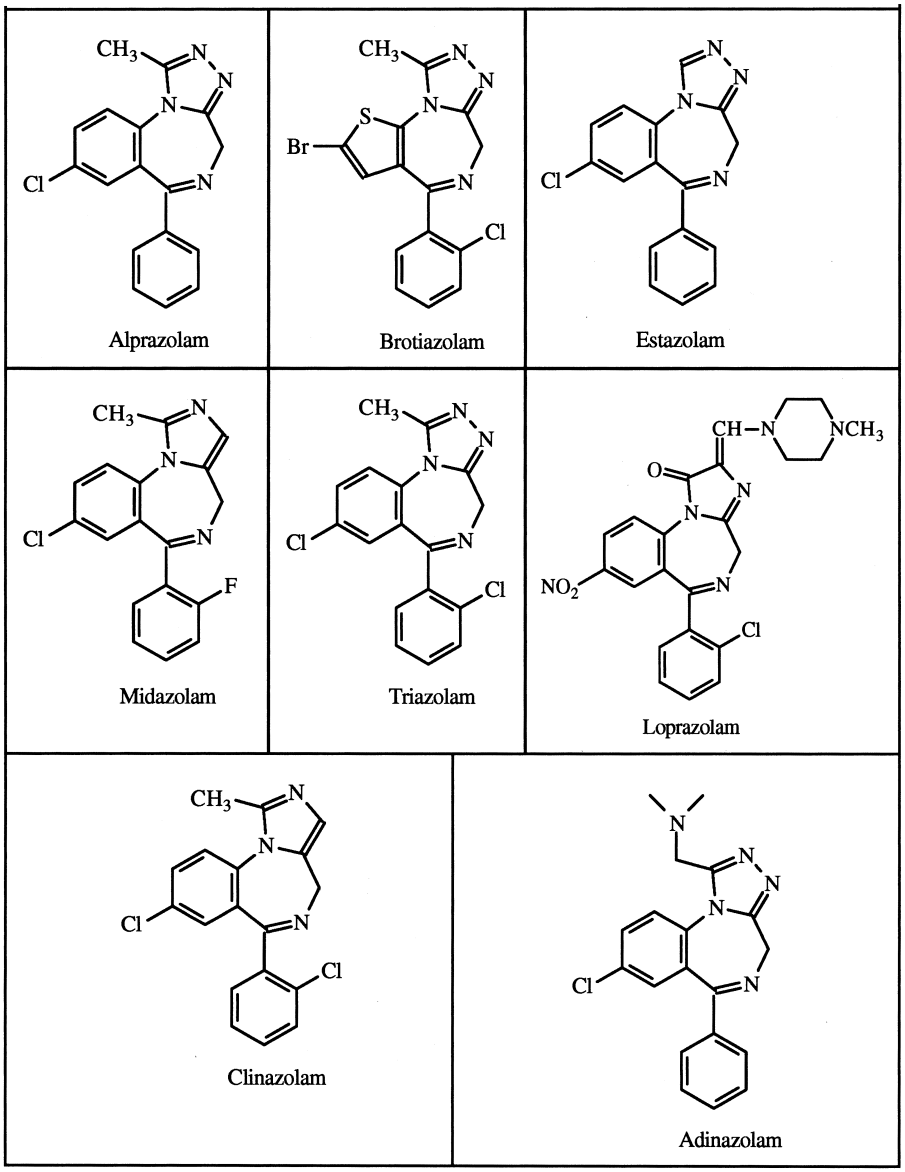


Fig. 2. Structures of imidazo- and triazolo-benzodiazepines.

4. Specimen preparation

4.1. Choice of specimen

The most common specimens used for the analysis of benzodiazepines are serum/plasma, blood, liver and urine. Blood, plasma and serum can often be interchanged in most methods, while urine may require hydrolysis prior to the isolation procedure.

In postmortem specimens the nitrobenzodiazepines are converted rapidly to the corresponding 7-amino metabolite, almost quantitatively [21]. Consequently, these 7-amino forms should be specifically targeted in postmortem cases.

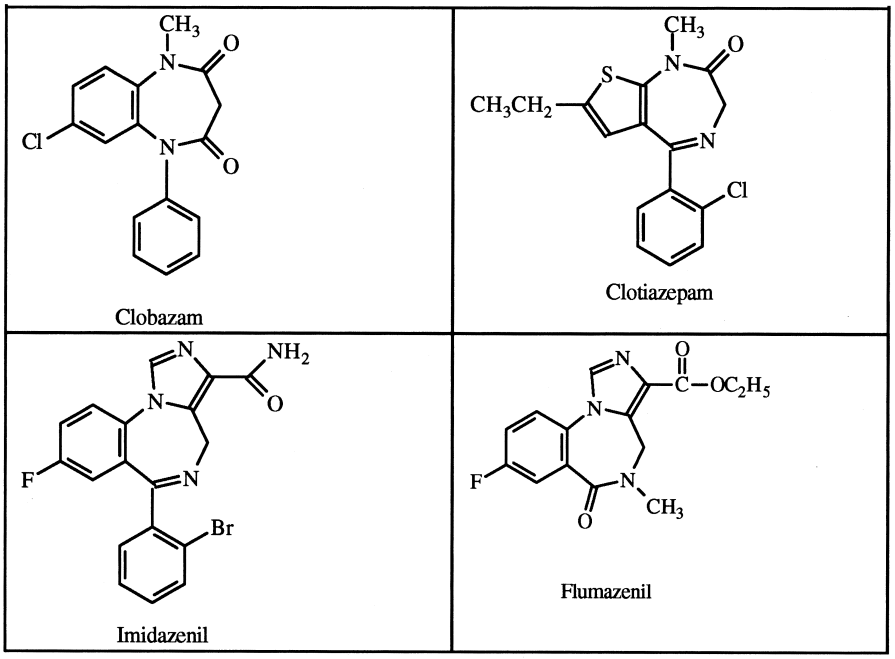


Fig. 3. Structures of miscellaneous benzodiazepines.

4.2. Hydrolysis conditions for urine

Meatherall [22,23] showed that prior hydrolysis of urine over 2 h at 56°C with *Helix pomatia* derived enzyme was optimum and this gave large increases in immunoreactivity with oxazepam, temazepam, lorazepam, diazepam and alprazolam, but not in subjects taking flurazepam. The use of 5000 units of *Helix pomatia* per ml of urine and incubation in acetate buffer at pH 4.5 was recommended.

Other authors have used similar procedures for the hydrolysis of urine [24–31], to mention a few. Variations include slightly lower or higher temperatures, the amount and source of enzyme used, the pH of buffer and time of incubation. When quantitative hydrolysis is required, it is recommended that individual variations be properly validated.

4.3. Nonchromatographic techniques for benzodiazepines

A number of nonchromatographic techniques are available for the analysis of urine and blood/plasma for the presence of benzodiazepines. These are frequently used to provide an initial test, or screen, of plasma or urine for the presence of benzodiazepines. Immunoassays are probably the most common class screening method for benzodiazepines [6,10,22,31–51]. Radioreceptor assays have also been reported for benzodiazepines [52–57].

A number of different immunoassay methods are available for benzodiazepines. Numerous commercial kits now exist for this purpose. These include enzyme immunoassays (EIA) (e.g. EMIT®) and enzyme linked inmmunosorbent assays (ELISA), fluorescent immunoassays (FPIA) (e.g., Abbott TDx™ and ADx™), agglutination or kinetic interaction of microparticles immunoassays (e.g., TRIAGE® and ONLINE™), cloned enzyme donor immunoassay (CEDIA®) and radio immunoassays (RIA) (DPC assays). These tests predominantly recognise the unconjugated benzodiazepines such as oxazepam and nordazepam. For this reason the sensitivity will not only be dependent on the cross-reactivities of the antibodies to the benzodiazepines, but also to the profile of metabolites present in urine, and the amount of target drug. Different batches of antibody will also influence the sensitivity and

selectivity to benzodiazepines and their metabolites. The TRIAGE assay uses two monoclonal antibodies, one raised at 3-position of the heterocyclic ring of 1,4-benzodiazepines and the other directed to hydroxylated and conjugated benzodiazepine metabolites at the N^1-position to improve its ability to detect a wide range of benzodiazepines [32].

It is beyond the scope of this review to provide detection limits for all benzodiazepines for all commercially available kits, however Table 2 summarises the outcomes of published studies using immunoassays and related tests.

Fitzgerald et al. [32] compared six commercial urine benzodiazepine immunoassays – Syva EMIT® d.a.u.™, Syva EMIT® II, Roche Abuscreen® ONLINE™, Abbott TDx™, Diagnostic Products Corp. (DPC) double-antibody radio immunoassay and Biosite TRIAGE™. All kits were successful in detecting oxazepam-related benzodiazepines while the DPC radio immunoassay and the TRIAGE kit were also able to detect lorazepam.

Fraser and Meatherall [33] also evaluated five commercial kits; the EMIT® d.a.u.™ assay, EMIT® II assay, Abbott TDx® assay, Bio Site TRIAGE™ device and Boehringer–Mannheim/Microgenics CEDIA® assay following single oral doses of alprazolam (0.5 mg) and triazolam (0.25 mg). They concluded that triazolam EMIT II gave the highest number of true positives with a screening cut-off of 100 ng/ml and a confirmation cut-off of 25 ng/ml, while the CEDIA assay was not sensitive to triazolam since its antibodies had a poor immunoreactivity to α-hydroxy triazolam. The TDx assay was less sensitive to the detection of these two benzodiazepines. TRIAGE did not detect these two drugs in any of the urine specimens. The authors concluded that the screening cut-off for these two drugs should be 100 ng/ml with a confirmation cut-off of 25 ng/ml (GC–MS).

The TRIAGE™ panel for drugs of abuse uses monoclonal antibodies which recognise glucuronide conjugates of benzodiazepines. This assay kit was found to have a sensitivity of 97.5% and a specificity of 94.3% over 326 urine specimens using a cut-off of 300 ng/ml. This device gave a higher detectability than both EMIT EIA and Abbott FPIA assays [34]. The benzodiazepines detected included oxazepam, temazepam, nordazepam, α-hydroxy alprazolam, lorazepam, desalkyl flurazepam, α-hydroxyethyl flurazepam and α-hydroxy triazolam.

An evaluation of the EMIT and FPIA urine screening assays was conducted for the detection of benzodiazepines following standard doses of a number of benzodiazepines [35]. The authors concluded that the detectability of the EMIT method was poor for flunitrazepam, lorazepam and nitrazepam, while the FPIA method gave poor detectability for chlordiazepoxide, flunitrazepam, lorazepam, nitrazepam and triazolam. However, the use of lower cut-offs and the use of enzymatic hydrolysis for FPIA substantially improved the detectability of all drugs.

A comparison of the CEDIA (Boehringer–Mannheim) and ONLINE (Roche) assays for benzodiazepines and other drugs of abuse suggested cut-offs of 200 and 100 ng/ml, respectively [36].

ELISA offers the advantage of automation away from a centralised laboratory using microtitre plates [37].

A serum kit (Abbott TDx or ADx) based on fluorescent polarisation immunoassay was capable of detecting the more potent benzodiazepines lorazepam, adinazolam and *N*-desmethyl adinazolam [38], alprazolam [39], α-hydroxy triazolam [40] and midazolam [41]. The EMIT assay was also capable of detecting adinazolam and its major metabolites [16] and midazolam and its α-hydroxy metabolite [41].

The use of prior hydrolysis of urine (see Section 4.2) increased the sensitivity to many of the benzodiazepines, particularly those metabolised to oxazepam [10,22,31,33,35,42]. However, Fitzgerald et al. [32] claimed that pretreatment of urine with glucuronidase was not necessary to detect oxazepam-related metabolites particularly if the cut-off levels were lowered.

Most kits designed for the detection of benzodiazepines are only applicable to urine. Since benzodiazepines may be present in urine and not in blood, direct tests on blood/plasma are useful to ascertain possible adverse influences of benzodiazepines on behaviour. Direct analysis on sera is preferable to a precipitation method, although the low-dose benzodiazepines flunitrazepam and triazolam, or those with poor cross-reactivity such as bromazepam and brotizolam, are not always detected [43].

Huang et al. [44] evaluated two RIA assays; the

Table 2
Summary of studies using immunoassay methods for the initial detection of benzodiazepines

Reference[a]	Specimen type[b]	Assays used	Specificities and conclusions
[35]	U untreated vs. hydrolysed	EMIT EIA and Abbott ADx FPIA	Standard doses of benzodiazepines given to volunteers and urine collected for 32 h. EMIT did not detect 1 mg flunitrazepam, 3.75 mg lorazepam and 5 mg nitrazepam, while Abbott FPIA did not detect 25 mg chlordiazepoxide, flunitrazepam, lorazepam, nitrazepam and 0.25 mg triazolam. Inclusion of a hydrolysis step and use of a lower cut-off improved detectability for FPIA.
[40]	U untreated	Abbott TDx FPIA and EMIT d.a.u. EIA serum assay	Both immunoassays were capable of detecting therapeutic triazolam usage in volunteers. Cross-reactivity for α-OH triazolam high for both assays.
[44]	1.0 B with BuCl extraction	Abuscreen RIA, DPC serum RIA assay, EMIT d.a.u. and EMIT tox serum EIA assays, X-systems urine and serum FPIA assays	Authors showed that these commercial immunoassays could be used to detect nordazepam, triazolam, lorazepam and alprazolam in blood particularly after prior extraction of blood with a solvent such as butyl chloride.
[43]	0.3 P direct and after acetone treatment	Abbott ADx serum assay, and ADx urine FPIA assays	Serum assay more sensitive than urine assay following acetone precipitation of plasma. Flunitrazepam and triazolam only suited to detection with serum assay.
[16]	U untreated	EMIT d.a.u. EIA and Abbott TDx urine FPIA assays.	Both assays capable of detecting adinazolam use in volunteers for 24 h using 300 ng/ml cut-off. Metabolites cross-reactive including *N*-desmethyladinazolam and estazolam.
[22]	U untreated vs. hydrolysed	EMIT II and Abbott TDx urine FPIA assays	Hydrolysis required for adequate detection of oxazepam, temazepam and lorazepam for both methods. A 100 ng/ml cut-off required for lorazepam screening. Flurazepam detected by both methods using 100 ng/ml cut-off. Alprazolam more sensitive on EMIT and after hydrolysis.
[32]	U untreated	EMIT d.a.u., EMIT II EIAs, Roche Abuscreen On-Line RIA, Abbott TDx FPIA, DPC RIA and Biosite Triage	All capable of detecting oxzazepam-related benzodiazepines. DPC RIA and Triage detected lorazepam. Hydrolysis not required to detect benzodiazepines when cut-offs were lowered.
[34]	U untreated	Triage, Abbott TDx FPIA and EMIT EIA	Triage detected oxazepam, nordazepam, temazepam, α-OH alprazolam, lorazepam, desalkylflurazepam, α-OH ethylflurazepam and α-OH triazolam in unhydrolysed urines. Senstivity and specificity were 97.5 and 94.3%, respectively. EMIT and TDx performed with lower sensitivity, or specificity, or both.
[31]	U untreated vs. hydrolysed	Abbott ADx urine FPIA	Hydrolysis increases sensitivity in patients taking oxazepam. Optimal conditions on 0.2 ml urine using glucuronidase from Escherichia coli were 500–1000 U/l for 30 min at 25°C
[10]	1.0 B and BuCl extraction	Abuscreen and DPC RIAs, X-systems serum and urine FPIAs, EMIT TOX serum and urine EIAs	Alprazolam, α-OH alprazolam, norclobazam, diazepam, estazolam, flunitrazepam, norflunitrazepam, midazolam, 4-OH midazolam, nitrazepam, noradinazolam, oxazepam, prazepam, 3-OH prazepam, temazepam and triazolam detected at 70 ng/ml. 7-Amino clonazepam, clonazepam, haloxazolam, loprazolam, 7-amino nitrazepam, and oxazolam not detected. Bromazepam, chlordiazepoxide, demoxepam and 4-OH alprazolam detected by RIA/EIA. Adinazolam detected by Abuscreen RIA, EIAs and FPIAs only. Clonazepam was detected by Abuscreen RIA and EIAs. Bentazepam, clotiazepam and medoxolam detected by EIAs and FPIAs. 7-Amino flunitrazepam and quazepam by EIAs only and α-OH triazolam and medazepam by Abuscreen RIA only.
[38]	P	Abbott TDx FPIA serum assay	Detected lorazepam at concentrations >25 ng/ml and adinazolam and *N*-desmethyladinazolam >50 ng/ml. Precision (between run) ranged was <7% in range 75–700 ng/ml nordazepam.
[67]	P	Abbott TDx serum FPIA assay and HPLC comparison	Showed that the FPIA assay detected both midazolam and α-OH metabolite, and that FPIA method overestimated the sum of these 2 species by 10% in patients receiving midazolam.

Table 2. Continued

Reference[a]	Specimen type[b]	Assays used	Specificities and conclusions
[33]	U untreated vs. hydrolysed	EMIT d.a.u., EMIT II EIAs, Abbott TDx FPIA, Biosite Triage and Boehringer Mannheim/Microgenics CEDIA	Recommended to lower cut-offs for alprazoplam and triazolam to 100 ng/ml and to include a hydrolysis step. CEDIA not sensitive to triazolam since no cross-reactivity to α-OH triazolam. Triage device did not detect ingestion of alprazolam (0.5 mg) and triazolam (0.25 mg) at all.
[37]	U untreated	ELISA	Antibody raised to oxazepam. Detection limit 0.3 μg/ml using 10 μl sample. Poor cross-reactivity to clonazepam, medazepam, lorazepam, flunitrazepam and clobazam and no cross-reactivity to chlordiazepoxide and flurazepam.
[49]	U untreated	Improved CEDIA assay and EMIT II assay	Improved CEDIA assay showed no significant cross-reactivity with sertraline metabolites and showed similar performance to EMIT assay.
[51]	1.0 B following extraction	Specific RIA for flunitrazepam	Detection limit 0.1 ng/ml, metabolites of flunitrazepam and other benzodiazepines did not cross-react with antibody.
[45]	0.1 P following extraction	Abbott TDx FPIA	Quantified lorazepam concentrations in plasma/sera after extraction with *t*-butyl methyl ether, detection limit 50 ng/ml, between run precision <9.0% and good correlation with HPLC assay.
[52]	0.02 P untreated	RRA using ^{3}H-flunitrazepam	Detection limit 5 ng/ml for clonazepam, triazolam (10), nitrazepam (20), lorazepam (25), alprazolam (70), diazepam (150), oxazepam (300), chlordiazepoxide (3500).
[53]	0.05 U untreated	RRA using ^{3}H-flunitrazepam and EMIT d.a.u assay	Hydrolysis improves response for conjugated benzodiazepines such as oxazepam, detection limits for RRA lower than for EMIT assay; detection limits using RRA are alprazolam (0.015 μ*M*), diazepam (0.07 μ*M*), flunitrazepam (0.02 μ*M*), lorazepam (0.02 μ*M*), nitrazepam (0.10 μ*M*), temazepam (0.10 μ*M*), oxazepam (0.5 μ*M*) and triazolam (0.00354 μ*M*).
[54]	0.5 P following extraction	RRA using ^{3}H-flunitrazepam	Obtained sera concentrations of diazepam-equivalents following therapeutic doses of a large range of benzodiazepines.
[55,57]	0.025 U untreated	RRA using ^{3}H-flunitrazepam	Showed applicability of RRA to systematic toxicological analysis.
[56]	0.5 U treated and untreated	RRA using ^{3}H-flunitrazepam, CEDIA and Roche ONLINE	Showed diagnostic sensitivity and specificity of RRA were almost equal or superior to CEDIA and ONLINE immunoassays, particularly for lorazepam which was not detected by immunoassays. Hydrolysis of urine was not considered necessary for detectability.

[a] References are chronologically listed.
[b] Specimen volumes are in ml.

Abuscreen and Diagnostic Products Corporation (DPC) serum kit, two EIA assays; the EMIT d.a.u. and EMIT TOX serum assay, and two FPIA assays; the X-systems urine and serum assays for their ability to detect benzodiazepines in fortified blood. The drugs studied were nordazepam, triazolam, lorazepam and alprazolam. They showed that these commercial immunoassays could be used to detect benzodiazepines in blood, particularly, if prior extraction of blood with a solvent such as butyl chloride was conducted. In later studies on actual blood specimens, the RIAs designed for blood/serum tended to perform better than those designed for urine, although this trend did not apply to all benzodiazepines [10]. For example, clonazepam was only detectable at 70 ng/ml by the Abuscreen RIA and the EMIT assays, while the three clonazepam metabolites were not detectable by any of the assays.

Prior extraction of serum can lead to a more accurate measurement of lorazepam concentrations in clinical studies using fluorescent polarisation immunoassay, since glucuronide metabolites which

partially cross-react with the antibody are selectively removed [45]. This presumably would apply to other benzodiazepines.

False positive results with benzodiazepine immunoassays have also been reported with the non-steroidal antiinflammatory drug oxaprozin (Syva EMIT II) [46,47] and with sertraline (CEDIA) [48,49]. Recently, interference by sertraline with the CEDIA kit has been eliminated by a change in the antibodies used [49,50].

Immunoassays for specific benzodiazepines have been described. West et al. [51] described a specific radio immunoassay for flunitrazepam after a flunitrazepam-glutaric acid hapten coupled to bovine serum albumin produced antisera in rabbits. Specificity for flunitrazepam was high with little cross-reactivity with metabolites. The detection limit from blood was <0.1 ng/ml.

Radioreceptor assays (RRA) have been published in recent years [52–56]. These show useful results can be obtained on a small sample volume with a high sensitivity. Clonazepam and triazolam, for example, can be measured down to 5 and 10 ng/ml, respectively, using 20 μl of specimen [52]. Detection limits were lower than those specified for the EMIT d.a.u. [53]. RRA has been shown to be as good or superior to the CEDIA assay and ONLINE agglutination immunoassays, particularly in that lorazepam was detected by the RRA and not by the immunoasays [56].

This RRA technique also has the advantage that all benzodiazepines and their pharmacologically active metabolites can be detected with equal sensitivity since the assay is based on a direct competition with benzodiazepine receptors. Sensitivity can be increased by hydrolysis to convert all glucuronides to the active form [53], however this was not considered necessary in real life [56].

The benzodiazepine antagonist flumazenil is not detected with FPIA and EIA [58] but can be detected by radio receptor assays [52].

5. Extraction techniques for benzodiazepines

Chromatographic techniques, with few exceptions, require some form of isolation procedure to separate the benzodiazepines from biological matrices. These procedures can be separated into three distinct types:

1. liquid–liquid extraction,
2. solid-phase extraction and
3. direct introduction into a chromatographic system.

5.1. Liquid–liquid extraction

Solvents used to extract benzodiazepines include diethyl ether [8,59–67], toluene [16,68,69], dichloromethane [26,70,71], butyl chloride [10,44,72,73], diethyl ether–2-propanol (70:30, v/v) [74], hexane–dichloromethane (50:50, v/v) [75], butyl chloride–ethyl acetate (20:80, v/v) [27], toluene–2-pentyl alcohol (98.5:1.5, v/v) [76], dichloromethane–diethyl ether (40:60, v/v) [62], chloroform–ethyl acetate (4:1, v/v) [77], butyl acetate [78,79], toluene–ethyl acetate [80], toluene–dichloromethane [81–84], butan-2-ol–dichloromethane [85], dichloromethane–butyl chloride [86] and diethyl ether–chloroform [87]. There is little to distinguish these solvents, with recoveries well over 60% for most benzodiazepines. Diethyl ether, however, is disadvantaged by its volatility and inherently high danger from fire.

An acid back-extraction has been used to clean-up extracts for HPLC assays [8], but this does not seem to offer any particular advantage over the use of solvents alone.

Extractions are most commonly conducted under slightly alkaline conditions by the use of sodium carbonate, phosphate, borate or tetraborate, Trizma buffer (pH 9–10). Dilute sodium hydroxide, or near neutral or unbuffered conditions are also described. There is little difference in performance of HPLC, GC and GC–MS techniques with these buffering agents (see Section 6).

5.2. Solid-phase extraction

Solid-phase approaches to the extraction of benzodiazepines include the use of Extrelut for HPLC assays [9,59,70]. Octadecylsilane-bonded cartridges (C_{18}) were commonly used [88–99], however C_2-bonded columns [90,100–102] were also successfully used. Mixed phase Bond-Elut Certify® were also

commonly used, particularly for GC applications [25,34,53,85,103–106]. Casas [90] studied the extractability and cleanliness of a number of solid-phase extraction columns. They concluded that the C_2 column provided the best combination of high recovery and clean extracts from urine, compared to C_8, C_{18}, phenyl and cyclohexyl phases, whilst CN provided little retention on the cartridge due to its polar nature.

Chen et al. [107] provided a drug screening method using the fully automated Gilson ASPEC® solid-phase extraction method for plasma and whole blood. This method used Bond-Elut Certify columns. Recoveries of ketazolam, lorazepam and oxazepam were better than 80% using acetone–chloroform (1:1, v/v) as eluent. A similar system was used satisfactorily for clobazam and its metabolite using Bond-Elut C_{18} cartridges [99].

Huang et al. [104] described a method for the isolation of benzodiazepines and other drugs from 100 mg of liver homogenate also using Bond-Elut Certify columns. Recoveries for diazepam and flunitrazepam were over 80%.

5.3. Direct injection

Four publications using HPLC to chromatograph the benzodiazepines described the use of a direct injection technique in which the benzodiazepines are preferentially absorbed onto a precolumn which are then back-flushed into the analytical column using column-switching [108–110] or following a dialysis pretreatment on-line [111]. While these techniques avoid an extraction step they do require more instrumentation. Its main advantage over other reported techniques are potential time savings. These techniques are however restricted to the use of HPLC.

6. Chromatographic techniques

In the context of this review there are four principle chromatographic procedures used to separate and detect benzodiazepines from each other and other components in extracts. These are HPLC, GC, TLC and micellar electrokinetic capillary chromatography (MECC).

Chromatographic methods can either be used to screen blood/plasma/serum and urine for the presence of one or more benzodiazepines, or confirm the presence of one or more benzodiazepines following an initial immunoassay or other screening test.

6.1. High-performance liquid chromatography

A summary of the HPLC methods reviewed here are shown in Table 3. Of the 42 methods, 8 were devoted specifically to urine, 28 to blood or plasma/serum, 5 to both specimen types and 1 to blood and liver. With the exception of one paper, all used reversed-phase separation, of which 25 used C_{18}, 8 used C_8, 3 used CN and 2 used phenyl. The commercial Remedi system was used in four papers [30,95,112,113].

UV absorbance detection was used by all, with the exception of two, which used LC coupled to MS–MS [59,97,106], and one which converted benzodiazepines to fluorescent derivatives with acetic acid as a post column reaction [100].

Photodiode-array detection (to supplement UV absorbance detection) offers real advantages to analysts in identifying peaks and assisting in establishing peak purity, however this was only described by three papers [88,96,98]. Photodiode array detection can be a very useful technique if GC and GC–MS instrumentation is not readily available or if absolute proof of structure is not required [115,116].

Detection limits hovered around 10–20 ng/ml, although this depended on the benzodiazepine measured (extinction coefficient), the volume of specimen extracted, the detection wavelength and the method used. A detection limit down to 0.5 ng/ml was reported for lorazepam using 1 ml of plasma and UV detection at 230 nm [101]. This method required almost all of the extract to be injected on-column. Detection limits of 1–2 ng/ml were also reported for a number of benzodiazepines [65,71,88,96,97,103]. Again these tended to either extract 2 ml of specimen and/or inject a large fraction of the extract. Most other methods tended to inject lesser fractions of the extract ranging from 1/2 to 1/12 of reconstituted residue after solvent evaporation. The use of small volumes of urine (0.1 ml) afforded sufficient sensitivity for urine testing for the presence of benzodiazepines [79].

Table 3. Summary of published HPLC methods

Reference[a]	Tissue[b]	Drugs	Extraction	Relative injection volume[c]	Column	Mobile phase	Flow rate[d]	Detection method[e]	Detection limit[f]	Recovery[g]
[70]	3.0 P	Alprazolam, triazolam	Extrelut extraction with DCM, and column switching	1/4	Precolumn PRP-1, 3 cm×2.1 mm I.D., 10 μm Column RP-8 Spheri-5, 10 cm× 4.6 mm I.D., 5 μm	ACN/water/phosphoric acid, isocratic	1.0	UV 230	10	86
[88]	2.0 B, S, PM-B	Numerous (8)	Acetone precipitation, and SPE C_{18} end-capped	1/2	Kontrosorb 10 RP-18 column, 250 mm×4.6 mm I.D.	ACN–phosphate, pH 2.3 (156:340), isocratic	1.3	DAD 190–430	2	>75
[59]	1.0 B	Numerous (10)	Extrelut, diethyl ether	1/5	Nova-Pak C_{18} 150 mm×3.9 mm I.D., 4 μm size	MeOH–NH_4Ac (60:40) isocratic	0.4	MS–MS	>50	>60
[60]	1.0 P	Midazolam	NaOH, diethyl ether with back extraction	1/5	μBondapak C_{18}, 300 mm×3.9 mm I.D.	ACN–0.05 *M* phosphate, pH 4.45, isocratic	1.0	UV 210	<5	>70
[111]	0.1 P	Diazepam nitrazepam oxazepam	On-line dialysis	All	C_{18} RoSil 150 mm× 3.1 mm I.D., 5 μm	MeOH–20 m*M* acetate buffer, pH 5.0, isocratic	0.5	UV 254	>20	37–50
[9]	2.5 U	Numerous (9)	SPE Extrelut/1O% isopropanol chloroform, ether clean-up	1/5	LiChrospher 100 RP8, 250 mm×4 mm ID., 5 μm	0.01 *M* phosphate, pH 3.5, 0.02 *M* MSA, ACN (various gradients)	1	UV 234	100	n/a
[89]	0.5 P	Numerous (7) C_{18}	SPE Bond Elut	1/2.5	Bakerbond C_8, 250 ×4.6 mm I.D., 5 μm	ACN–MeOH–water–perchloric acid 95:1:4.5:0.0015), isocratic	2.0	UV 230	>10	>65
[16]	2.0 U	Adinazolam (as *N*-desmethyl metabolite)	Na_2CO_3 Toluene	1/12	RP-8 25 cm×4.6 mm I.D., 5 μm	0.01 *M* phosphate–ACN–nonylamine (800:200:0.5), isocratic	1.6	UV 230	100	86
[100]	1.0 U	Oxazepam	SPE Bond-elut C_2	1/50	Ultrabase C_{18}, 3.5 cm×4.6 mm I.D., 5 μm	MeOH–water (60:40), isocratic	0.5	F364/469 (acetic acid)	4	>95
[8]	0.5 PM-B	Numerous (15)	Na_2CO_3 diethylether and acid clean-up step	1/4	Nova-Pak phenyl, 15 cm×4.6 mm ID., 5 μm	15–25% ACN/phospate, pH 3.8, gradient	0.8	UV 240	50	14–79
[101]	1.0 P	Lorazepam	SPE Bond-Elut C-2	1/1.2	APEX octadecyl 25 cm×4.6 mm I.D., 5 μm	ACN–0.05 *M* phosphate, 20 m*M* TEA, pH 7.0 (33:67), isocratic	2.0	UV 230	0.5	75

Table 3. Continued

Reference[a]	Tissue[b]	Drugs	Extraction	Relative injection volume[c]	Column	Mobile phase	Flow rate[d]	Detection method[e]	Detection limit[f]	Recovery[g]
[61]	0.1 P	Bromazepam	Na_2CO_3 diethyl ether	1/5	Nova-Pak C_{18}, 15 cm×3.9 mm I.D., 5 μm	ACN–water–TEAP, pH 7.4 (300:700:4), isocratic	2.0	UV 240	50	100
[91]	1.0 P	Diazepam/ nordazepam	SPE Bond-Elut C_{18}	1/4	μBondapak C_{18}, 15 cm×3.9 mm I.D., 10 μm	MeOH–30 m*M* phosphate, pH 3.5 (55:45), isocratic	1.5	UV 229	5	>98
[108]	0.1–0.2 P	Clobazam, diazepam	Column switching, direct injection	All	Ultrasphere ODS, 75 mm×4.6 mm I.D., 3 μm	ACN–phosphate, 65 mM–1% TEA, pH 5.4 (33:67), isocratic	1.0	UV 230	20	>92
[103]	1.0 P	Flunitrazepam and metabolites	SPE Bond-Elut Certify	2/5	C_{18} Select-B, 25 cm×4 mm I.D., 5 μm	ACN–0.02 *M* phosphate, pH 2.0, isocratic	1.0	UV 254	1	>90
[90]	1.0 P, U	Numerous (7)	Various SPE procedures (C_2, CN, C_{18}, C_8, Ph, CH)	1/10	Ultrabase C_{18}, 35 ×4.6 mm I.D., 5 μm	MeOH–water (60:40), isocratic	Various	UV various	>60	upto >90
[92]	1.0 P	Flunitrazepam	SPE Superclean LC-18, pH 6.9	1/10	Novapak C_{18}, 150 ×3.9 mm I.D., 5 μm	ACN–10 m*M* ammonium acetate, pH 6.7 (45:55), isocrtic	1.0	UV 250	10	>85
[62]	0.5 P, U	Midazolam, flumazenil and metabolites	Hydrolysis (urine) phosphate, pH 9 DCM–diethyl ether (60:40)	3/8	Novapak C_{18}, 100 ×8 mm I.D., 5 mm	ACN–0.04 *M* phosphate, 1% TEA, pH 7.2 (32:68), isocratic	1.5	UV 220	4–20	>80
[93]	1.0 P, U	Temazepam, oxazepam	SPE Bond-EIut C_{18}	1/5	LiChrospher 100 RP8, 125×4 mm I.D., 5 μm	ACN–0.01 *M* phosphate, pH 5.6, isocratic	1.6	UV 254	5	>70
[94]	1.0 P	Clonazepam	SPE, Extra-Sep C_{18}	1/2.6	Velosep C_{18}, 100 ×3.2 mm I.D.	ACN–0.5% acetic acid (32:68), isocratic	0.5	UV 306	5	90
[109]	0.6 P	Midazolam	Column switching, direct injection	All	LiChroSphere 60 RP-C_{18} select, 250 ×4 mm I.D.	ACN–phosphate, gradient	1.0	230	10	>80
[63]	0.05 P rat	Alprazolam and metabolites	Borate pH 9.0 diethyl ether	2/5	Ultrasphere C_{18}, 150×2 mm I.D., 5 μm	MeOH–ACN–43 m*M* acetate, pH 2.4 (45:8:47), isocratic	0.3	UV 230	5	>80
[114]	1.0 P	Midazolam and 2 metabolites	SPE Presep C_1	1/2	Spherisorb C_8, 100 ×4.6 mm I.D., 5 μm	ACN–MeOH–0.02 *M* phosphate, 0.2 *M* TBABr, pH 4.1, isocratic	1.5	UV 254	15	>80
[53]	2.5 U	Numerous	Hydrlysis, SPE Bond Elut Cerfity pH 5.2	1/5	LiChrospher 100 RP-18, 250×4 mm I.D., 5 μm	1. ACN–20 m*M* phosphate pH 5.4 (40:60), isocratic 2. ACN–2O m*M* phosphate pH 5.4, 5 m*M* TMAH, isocratic	1.0	UV 252	5	n/a

(Cont.)

Table 3. Continued

Reference[a]	Tissue[b]	Drugs	Extraction	Relative injection volume[c]	Column	Mobile phase	Flow rate[d]	Detection method[e]	Detection limit[f]	Recovery[g]
[64]	1.0 P	Flunitrazepam	NaOH diethyl ether	1/2.5	Novapak C_{18}, 150× 3.9 mm I.D., 5 μm	ACN–water–TEAP pH 7.5 (300:700:4), isocratic	2.0	UV 230	5	n/a
[95]	2.0 P	Numerous (8)	SPE Bond Elut C_{18}	n/a	Remedi (4 columns)	Remedi (Bio Rad), commercial system	n/a	DAD 193–305	~>500	~60
[110]	0.1 P	Diazepam	Direct injection, deproteinisation on a hydroxy apatite pre-column	All	Inertsil ODS-2, 150 ×4.6 mm I.D., 5 μm, 40°C	ACN–water (50:50), isocratic	1.0	UV 240	100	>90
[71]	1.0 U	Diazepam, nordazepam, oxazepam temazepam	Hydrolysis, Na_3PO_4 DCM	60/150	LiChrospber 100 RP8, 5 μm	Water–MeOH–TEAP, pH 3.5 (70:30:0.1), isocratic	0.7	UV 240	2	>82
[112]	2.0 B, L	Alprazolam and metabolite	Acetone precipitation and SPE	n/a	Bio-Rad C_8, 100× 2.1 mm I.D., 3 μm	ACN–0.01 *M* KH_2PO_4, 0.01% *N*,*N*-dimethyloctylamine (30:70), pH 6.4	0.3	UV 242	18	>70
[72]	0.5 ml B, P	Clonazepam, flunitrazepam, nitrazepam	Na_2CO_3 BuCl	1/4	Nova-pak phenyl 150×3.9 mm I.D., 4 μm	ACN–40 m*M* phosphate, pH 3.75 (28:72), isocratic	0.8	UV 230	10	>80
[65]	1.0 P	Midazolam 1-hydroxy metabolite	Borate pH 9.0 diethyl ether	n/a	Spherisorb CN, 150 ×4.6 mm I.D., 5 μm	MeOH–2-propanol with 0.015% perchloric acid (75:25) isocratic	1.5	UV 215	2	>85
[96]	1.0 P	Alprazolam, clonazepam nitrazepam	SPE Bond-Elut C_{18}	1/5	Novapak C_{18}, 4 μm	ACN–MeOH–10 m*M* phosphate, pH 3.7 (30:2:100), isocratic	1.5	UV 240 DAD 210–365	2	>90
[30]	1.0 U	Numerous	REMEDi system		Remedi (4 columns)	Remedi (BioRad), commercial system	n/a	DAD 193–305	Cut-offs used	n/a
[97]	1.0 P, U	Numerous (5)	SPE C_{18} Bond Elut and narc-2 Bakerbond	1/20	LiChrospher 60-RP Select B, 100×2.0 mm I.D., 5 μm	MeOH–ACN–water (1:1:1) isocratic	0.1	MS–MS	2	>90
[66]	0.2 P rat	Imidazenil, alprazolam	Diethyl ether	n/a	LiChrosorb CN, 250 ×4 mm I.D., 5 μm	1-Hexane–MeOH–acetic acid (78:17.6:4.4), isocratic	1.5	UV 255	30	~100
[98]	1.0 P; 0.1 U sheep	Oxazepam and glucuronides	SPE Bond-Elut C_{18}	1/5	LiChrosorb RP-18, 250×4 mm I.D., 5 μm	ACN–2-propanol–25% phosphoric acid (180:75:12) diluted to 1 l, pH 2.05, isocratic	1.4	DAD	>20	>85
[99]	1.0 P	Clobazam and metabolite	SPE Bond-Elut C_{18}	1/30	Novapak C_{18}, 150× 3.9 mm I.D., 4 μm	ACN–MeOH–10 m*M* potassium phosphate, pH 3.7 (30:2:100)	1.5	DAD 210–365	4	>97

Table 3. Continued

Reference[a]	Tissue[b]	Drugs	Extraction	Relative injection volume[c]	Column	Mobile phase	Flow rate[d]	Detection method[e]	Detection limit[f]	Recovery[g]
[87]	2.0 P	Flunitrazepam and four metabolites	NH_4Cl buffer Diethyl ether –chloroform (4:1)	4/5	RSil CN, 300×3.9 mm I.D., 10 μm	MeOH–30 m*M* phosphate, pH 4.0 (17:83)	1.6	UV 242	>2.5	30–83
[106]	1.0 B	Numerous (10)	SPE Bond-Elut Certify	1/5	Nova-Pak C_{18}, 150 ×3.9 mm I.D., 4 μm	MeOH–50 m*M* NH_4Ac (75:25)	0.6	MS–MS	0.05–0.5	~50
[113]	1.0 U	Demoxepam	n/a	n/a	Remedi system with direct probe MS of effluent	Remedi system (commercial)	n/a	DAD 220–265 and EI MS	500	n/a
[67]	1.0 P	Midazolam and metabolite	Diethyl ether	1/2	Nucleosil 250×4.0 mm I.D., 5 μm	MeOH–0.02 *M* sodium acetate, pH 7.4 (55:45)	1.2	UV 215	2	>55
[79]	0.1 U	Numerous (7)	Na_2CO_3 BuAc	–	HiChrom C_{18} RPB, 250×0.4 mm I.D., 5 μm	Water–MeOH–ACN–acetic acid (51:28:16:6), isocratic	1.0	UV 241	>100	>70

n/a – not available or not described.

[a] ted in chronological order.

[b] Volume of fluid in ml.

[c] Proportion of extract injected into HPLC.

[d] Flow-rate in ml/min.

[e] Wavelengths in nm.

[f] Detection limits in ng/ml.

[g] Recoveries in per cent.

Mobile phases used varied from methanol/unbuffered water to solvent/buffered phosphate solutions, the base modifier triethylamine and ion-pairing reagents such as methane sulfonic acid, tetramethyl ammonium hydrogen sulphate and tetrabutyl ammonium bromide. Three of the methods used gradient programming [8,9,109].

One method used normal-phase chromatography on a CN column [66] which separated the novel partial benzodiazepine agonist imidazenil from alprazolam which was used as internal standard.

McIntyre et al. [8] used a liquid extraction (diethyl ether with back extraction) and a gradient HPLC with photodiode array detection with a phenyl-bonded column to confirm blood specimens for the presence of benzodiazepines. This assay was fully validated with respect to accuracy, precision, reproducibility, detection limits for over 15 benzodiazepines and selected metabolites and gave data on possible coelution of many nonbenzodiazepine-related substances. Detection limits down to 10 μg/l were claimed. Variations of this method from the same laboratory showed that butyl chloride could substitute for diethyl ether and that the back-extraction step could be omitted without significant loss of performance [72].

Ferrara et al. [9] used solid-phase extraction (Extrelut) of hydrolysed urine and gradient HPLC with UV detection (234 nm) and a C_8 bonded-phase column to confirm benzodiazepine-positive specimens after an EMIT screen. Nine benzodiazepines or benzodiazepine metabolites were detected in an isocratic system.

Mußhoff and Daldrup [88] described a solid-phase extraction method and isocratic HPLC method with photodiode array detection to confirm the presence of eight benzodiazepines including bromazepam, diazepam, flunitrazepam, nordazepam, oxazepam and triazolam. The method used an acetone precipitation step prior to SPE (C_{18} cartridges) and was fully validated for blood and serum. Detection limits for triazolam, the most difficult benzodiazepine to con-

firm due to its very high potency, was 10 ng/ml if a UV spectral match was desired, or 1–2 ng/ml if detection was only required.

The REMEDi drug-profiling system is an automated HPLC system using a 4-column separation and extraction system for confirmation of urine specimens for a number of drugs including the benzodiazepines [95]. Demedts et al. [117] showed that its detection rate for benzodiazepines was less than 20%. Sensitivity of this system is relatively low and will therefore not confirm benzodiazepines at concentrations much less than 1000 ng/ml. Essien et al. [113] used direct probe EI MS of an extracted cut of a HPLC peak to confirm demoxepam following separation on a REMEDi system.

The combination of HPLC with tandem MS (LC–MS–MS) provides a good example of the separation power of HPLC with the sensitivity and specificity of MS. Detection limits for 10 benzodiazepines ranged from 10 to 200 pg on-column resulting in detection limits of better than 1 ng/ml for the benzodiazepines in whole blood using a thermospray interface [59]. Similar performance data on both plasma and urine (1 ml) for 5 benzodiazepines were given by Kleinschmidt et al. using an electrospray interface [97] and for a range of hypnotic and sedative drugs including 10 benzodiazepines using thermospray tandem MS [106].

6.2. Gas chromatography and gas chromatography–mass spectrometry

GC methods generally used fused-silica, although one used a deactivated metal capillary column [69]. The type of column ranged from low polarity dimethylpolysiloxane, 14% cyanopropylphenyl, 5% diphenyl methylpolysiloxane to the polar trifluoropropylpolysiloxane to 50% diphenyl methylpolysiloxane phases (see Table 4). Most methods utilised temperature programming. Due to the wide polarity differences, temperature programming is necessary for assays involving detection of a number of benzodiazepines.

The use of a cyanopropylphenyl stationary phase gave better separation of a number of underivitised benzodiazepines to a methylsilicone phase [78]. Similarly, the slightly polar 5% phenyl methylsilicone columns have been used to separate a number of underivitised benzodiazepines [73,85]. Derivitised benzodiazepines have either been separated on a 100% methylsilicone phase [24,25,28–30,34,40,75,80,86,104,119,120], or a 5% phenyl methylsilicone phase [26,27,81–85,68,73,105,118].

With the exception of three, all used solvent extraction, however, there was no common solvent system used including; ether–2-propanol (7:3, v/v), hexane–dichloromethane (1:1, v/v), toluene–2-pentyl alcohol (98.5:1.5, v/v), toluene, butyl chloride, butyl acetate and chloroform–ethyl acetate (4:1, v/v). Butyl chloride gave a wide range of recoveries (26–93%) for a number of benzodiazepines [73], triazolam was recovered quantitatively with toluene [69], butyl acetate gave recoveries of 79–98% [75], and the recovery for midazolam and flumazenil was better than 85% for chloroform–ethyl acetate (4:1, v/v) [77].

ECD detectors gave the best detection limits ($\leq$1 ng/ml) for 1.0 ml plasma [69,75,76,78], although NPD provided detection limits down to 5 to 25 ng/ml [118] and 20 ng/ml [73].

All of these methods injected neat benzodiazepines. Unfortunately, many benzodiazepines are thermally unstable and will produce multiple peaks, particularly if liners are not kept scrupulously clean [121].

One paper used a validated solvent-modified solid-phase microextraction for the analysis of diazepam in plasma specimens [119]. This is a novel approach used elsewhere for other substances and appears to have applicability for benzodiazepines. The thermal stability of other benzodiazepines will need to be defined before applicability to other members of this group can be shown.

Direct on-line injection using a dialysis technique involving a PLRP-S copolymer precolumn for absorption has also been reported on small sample volumes [118].

A number of methods utilising GC–MS were described. These are also summarised in Table 4. Again both liquid–liquid and solid-phase extraction methods have been successfully used. Recoveries were all 65% or greater. As expected, all used fused silica columns, ranging from 100% methylsilicone to mixed phenylmethylsilicone and trifluoropropylmethyl phases. Derivitisation of benzodiaze-

Table 4. Summary of published GC and GC–MS methods

Reference[a]	Tissue[b]	Drugs	Extraction method	Column/conditions	Derivitisation method	Detection method	Detection limit[c]	Recovery[d]
[74]	0.5 U	Diazepam, temazepam oxazepam and metabolites	Hydrolysis, ether–2-propanol (7:3)	FSC 50% PMS (DB-17), 15 m megabore, N_2 CG, T=225°C isothermal	None	ECD	125	n/a
[78]	0.5 B	Numerous (9)	BuAc	FSC 14% CNPP (DB-1701), 15 m×0.32 mm I.D., and DMS (DB-1), T=120–280°C gradient	None	ECD	1	79–98
[75]	1.0 P	Triazolam	Hexane–DCM (50:50)	FSC DMS (SPB), 15 m×0.25 mm I.D. 0.25 μm film, split 40:1, T=200–265°C gradient	None	ECD	1	n/a
[26]	3.0 U	Numerous and metabolite (10)	Hydrolysis, DCM	FSC 5% PMS (DB-5), 30 m×0.25 mm I.D., 0.25 μm film, T=130–300 gradient	MeI/THAHS	EI	13–25	49–95
[40]	2.0 U	α-OH-triazolam	Hydrolysis, Na_2CO_3 DCM	FSC DMS (DB-1), 15 m×0.26mm I.D. split 10:1, T=280°C isothermal	BSTFA/TMCS	EI	<300	n/a
[80]	20 P	Diazepam, nordazepam oxazepam	Toluene–ethyl acetate (4:1)	FSC DMS (SPB-1), 30 m×0.75 mm I.D., 1.0 μm film, splitless, T=250–280°C gradient	MTBSTFA	EI	0.001	n/a
[27]	5.0 U	Numerous and metabolites (6)	Hydrolysis, BuCl–ethyl acetate (1:4)	FSC 5% PMS (DB-5), 15 m×0.32mm I.D. 0.25 μm film, splitless, T=210–300°C gradient	MTBSTFA	EI	10	>90
[76]	1.0 P	Estazolam	Toluene–2-pentyl alcohol or benzene–2-pentyl alcohol (98.5:1.5)	FSC 1.2 m×4 mm I.D. 3% SP-2250 on 80/100 Supelcoport, carrier Ar–Methane CG, T=280°C isothermal	None	ECD	1	n/a
[68]	0.2 P	Clobazam	Toluene	FSC 5% PMS (HP-5), 5.0 m×0.53 mm I.D., 5 μm film, T=250°C isotbermal	None	ECD	70	88
[28]	U	Numerous and metabolites (7)	Hydrolysis, Toluene–hexane–2-pentyl alcohol (78:20:2)	FSC DMS (DB-1), 15 m×0.32 mm I.D. 0.25 μm film, splitless, T=160–280°C gradient	BSTFA/TMCS,	EI, PI, CI	10–50	>70%
[25]	2.0	Numerous (5)	Hydrolysis, SPE Certify columns	FSC 12.5 m DMS (HP-1), splitless, T=120–310°C gradient	MTBSTFA	EI	1	73–83
[73]	1.0 B, P	Numerous and metabolites (10)	BuCl	FSC 5% PMS (BP-5), 12 m×0.53 mm I.D., 1 μm film, splitless, T=100–310°C gradient	None	NPD	20	26–93
[81]	0.2 P mouse	Chlordiazoxide, halazepam	Toluene–DCM (70:30)	FSC 5% PMS (HP-5), 25 m×0.25 mm I.D., 0.33 μm film, T=180–300°C gradient	BSTFA/TMCS	NCI	0.1	n/a
[82]	0.1–0.2 P mouse, rat	Flurazepam and metabolites	Toluene–DCM (70:30)	FSC 5% PMS (HP-5), 25 m×0.25 mm I.D., 0.33 μm film, T=180–300°C gradient	BSTFA/TMCS	NCI	0.1–0.5	>90
[105]	5.0 U	Numerous and metabolites (5)	Hydrolysis, SPE Bond-Elut Certify columns, pH 4–5	FSC 5% PMS, 25 m×0.2 mm I.D., 0.33 μm film, split 50:1, T=240–300°C gradient	BSTFA/TMCS	EI	<100	>65
[24]	2.0 U	Numerous and metabolites (8)	Hydrolysis, SPE SPEC.3ML.MP3, pH 9.5	FSC DMS (HP-1), 12 m×0.25 mm ID., 0.33 μm film, splitless, T=160–290°C gradient	BSTFA/TMCS	EI	25	>85

(Cont.)

Table 4. Continued

Reference[a]	Tissue[b]	Drugs	Extraction method	Column/conditions	Derivitisation method	Detection method	Detection limit[c]	Recovery[d]
[120]	1.0 U	Numerous and metabolites (10)	Hydrolysis, methyl-isobutyl ether–chloroform (2:1)	FSC DMS (DB-1), 15 m×0.25 mm I.D., 0.25 μm film, deactivated, T=140–290°C gradient	Propylation and propionylation	EI	5–25	>70
[29]	3.0 U	Numerous and metabolites (10)	Hydrolysis, Clean Screen DAU columns	FSC DMS (Ultra I), 12 m×0.2 mm ID., film 0.33 μm, splitless, T=100–300°C gradient	BSTFA/TMCS	EI	5–20	>80
[34]	2.0 U	Numerous	Hydrolysis, SPE Bond-Elut Certify columns	FSC DMS (HP-1), 12 m×0.2 mm I.D., 0.33 μm film splitless, T details not given	BSTFA	EI	n/a	n/a
[77]	1.0 P	Midazolam, flumazenil	Chloroform–ethyl acetate (4:1)	FSC TFPMS (RTx-5), 30 m×0.25 mm I.D., 0.25 μm film, T=300°C isothermal	None	NPD	3	>85
[102]	1.0 U	Flunitrepam, nitrazepam 7-amino metabolites	Hydrolysis, SPE Bond-Elut C_2	FSC TFPMS (Rtx-200), 30 m×0.25 mm I.D., 0.25 μm film, T=300°C isothermal	None	EI	>85	>85
[83]	1.0 P	Flumazenil	Toluene–DCM (70:30)	FSC 5% PMS (HP-5), 15 m×0.20 mm I.D., 0.33 μm film, splitless, T=130–300°C gradient	None	NCI	0.1	>99
[85]	2.0 U	Numerous and metabolites (9)	Hydrolysis 1. 2-Butanol–DCM (1:9) 2. SPE Bond-Elut Certify	FSC 5% PMS (DB-5), 12 m×0.25 mm I.D., 0.25 mm film, splitless, T=250–280°C gradient	None	EI	80	n/a
[118]	0.1 P	Numerous and metabolites (5)	On-line dialysis	FSC 5% PMS (SE-54), 15 m×0.32 mm I.D., 0.25 μm film, T=110–300°C gradient	None	FID/NPD	>5	14–41
[69]	1.0 P	Triazolam	NaOH, toluene	Metal capillary (OV-17) 50% PMS, 15 m ×0.25 mm I.D., 0.25 μm film, T=110–300°C gradient	None	ECD	0.5	100
[104]	0.1 L	Diazepam, flunitrazepam	SPE Bond-Elut Certify, 2-step extraction	FSC DMS (HP-1) 30 m×0.53 mm ID., 0.88 μm film, T=80–280 (FID), T=150–285 (NPD) °C gradient	None	FID/NPD	n/a	>80
[86]	>0.04 P	Midazolam	Na_3PO_4 DCM–BuCl (4:96)	FSC DMS (Ultra I), 12 m×0.2 mm I.D., 0.33 μm film, splitless, T=120–310°C gradient	None	EI	10	~90
[84]	1.0 P	Clonazepam	Borate, pH 10.0 Toluene–DCM (70:30)	FSC 5% DMS (HP-5), 12 m×0.25 mm I.D., 0.33 μm film, splitless, T=180–300°C gradient	BSTFA/TMCS	NCI	0.25	n/a
[30]	3.0 U	Numerous and metabolites (15)	Hydrolysis, Chloroform–2-propanol (9:1)	FSC DMS (HP-1), 12 m×0.25 mm I.D., 0.33 μm film, split 20:1, T=70–290°C gradient	BSTFA	EI	10–35	n/a
[119]	0.45 P	Diazepam	SPME	FSC DMS (DB-I), 30 m×0.2 mm I.D., 0.25 μm film, T=150–300°C gradient	None	NPD/FID	20	n/a

n/a – not available or not described.

[a] References are cited in chronological order.

[b] Volume of fluid in ml.

[c] Detection limits in ng/ml.

[d] Recoveries in per cent.

pines was preferred, which afforded better thermal stability and generally produced derivatives with well defined mass spectra. Derivitisation reagents ranged from the conventional TMS-derivative using BSTFA/TMCS [24,28–30,34,40,81,82,105,106], to *tert.*-butyldimethyl-derivatives [25,27,80], methyl [26], and combined propylated and propionylated derivatives [113]. Spectral characteristics for some selected benzodiazepines are shown in Table 5.

Mass spectra of benzodiazepines can be found in a number of references and books including Maurer and Pfleger [12]. McCarley and Brodbelt [122] provide a study of diagnostic ion–molecule reactions of some common 1,4-benzodiazepines.

Table 5
Mass spectral data for selected benzodiazepine and derivatives

Reference[a]	Derivative	Benzodiazepine	Ions monitored[b] (m/z and relative abundance)
[102]	None	7-Amino flunirazepam	283, 255(80), 254(58), 282(41)
		7-Amino nitrazepam	251, 222(97), 223(75), 105(23)
[26]	Methyl-EI	Alprazolam	256, 156, 221(27), 283(83), 284
		Oxazepam	271, 255(42), 273, 314(15)
		Lorazepam	305, 289(21), 291, 307(65), 313, 348
		α-Hydroxy triazolam	342, 343(39), 344(67)
		α-Hydroxy alprazolam	308, 309(25), 310(32)
		Nordazepam	256, 165, 221(27), 283(83), 284
[27]	*tert.*-Butyldimethyl silyl-EI	Oxazepam	462, 464(46), 519(28)
		Temazepam	357, 255(43), 283(54)
		Temazepam	491, 493(77), 513(83)
		α-Hydroxy alprazolam	381, 382(30), 383(41)
		α-Hydroxy triazolam	415, 41631), 417(72)
		2-Hydroxy ethylflurazepam	389, 345(11), 391(40)
[28]	Trimethylsilyl-NCI	Nordazepam	234, 269(22), 254(18), 342(15)
		Oxazepam	268, 270(34), 269(18), 271(6)
		Lorazepam	302, 303(17), 304(68), 305(12)
		Temazepam	372, 374(42), 373(29), 282(12)
		α-Hydroxy alprazolam	396, 398(41), 397(33), 399(11)
		α-Hydroxy triazolam	394, 396(32), 395(41), 397(11)
		N-1-Hydroxy ethylflurazepam	404, 405(42), 406(42), 403(28)
[105]	Trimethylsilyl-EI	Oxazepam	429, 415(18), 401(20)
		Lorazepam	429, 430(33), 432(13)
		7-Amino clonazepam	429, 394(99), 414(30)
		α-Hydroxy alprazolam	381, 382(30), 396(40)
[120]	Propylpropionate-EI	Diazepam	283, 256(85), 285(51), 257(48)
		Nordazepam	269, 311(93), 313(67), 284(66)
		Temazepam	271, 273(34), 357(29), 300(20)
		Oxazepam	299, 257(35), 301(30), 300(18)
		Lorazepam	333, 335(74), 57(40), 334(40)
		α-Hydroxy alprazolam	323, 324(46), 325(38), 381(25)
		α-Hydroxy triazolam	357, 359(68), 358(45), 36(30)
		N-Desalky flurazepam	287, 331(84), 329(76), 288(66)
		7-Amino flunitrazepam	39, 338(90), 311(77), 255(41)

[a] References in chronological order.
[b] Base ion as 100% with next best 2 or 3 ions (abundance in parentheses).

Meatherall [120] described a method to confirm a large number of benzodiazepines and metabolites using a conventional solvent extraction technique (methyl-*t*-butyl ether–chloroform, 2:1, v/v) following hydrolysis of 1 ml urine with glucuronidase (*Helix pomatia* at 56°C for 2 h). The N_1 position of extracted benzodiazepines was propylated with propyl iodide in TMAH (2.5%), while hydroxy groups were propionylated with propionyl chloride. The method was fully validated with respect to selection of ions, precision, extraction efficiency and limits of detection and quantitation. Derivatives show good spectral properties and chromatographic behaviour.

Joern [26] described a GC–MS method for a number of commonly encountered benzodiazepines using an extractive alkylation procedure based on the reaction of benzodiazepines with methyl iodide in dichloromethane and tetrahexyl ammonium hydrogen sulphate. Hydrolysed urine was initially extracted with dichloromethane prior to derivitisation. This method was validated, but there were some limitations. Oxazepam and temazepam formed the same derivative, two hydroxy metabolites of flurazepam were very difficult to derivatise and chlordiazepoxide and demoxepam were also difficult to detect.

The use of *t*-butyl-dimethylsilyl ethers of benzodiazepines, as a replacement for the conventional trimethylsilyl ethers, was described in three papers [25,27,80]. These derivatives offer excellent spectral characteristics for all common benzodiazepines including oxazepam, temazepam, lorazepam, α-hydroxy alprazolam, α-hydroxy triazolam and 2-hydroxyethylflurazepam. The *t*-butyl portion of the silyl ether is easily cleaved giving M−57 as the most intense ion.

The most frequent derivative described was the trimethylsilyl ether [24,28–30,34,40,81,82,105,106]. The use of negative ion chemical ionisation (NCI), with methane as reagent gas, afforded a greatly enhanced detection limit compared to electron impact mass spectrometry (EI-MS) [81–84]. In this NCI mode the loss of HCl occurred producing a signal ion at M−36, with a single ion isotopic cluster of A+1, A+2, A+3 and A+4. The mass spectrometer run in this mode afforded a much lower detection limit of ~0.1 ng/ml compared to positive EI and positive CI (generally ~0.1 ng/ml).

NCI-MS has also been used for the determination of flunitrazepam and 7-amino flunitrazepam (HFB derivatives) [123], nordazepam (TMS derivative) [124] and alprazolam and α-hydroxy alprazolam (TMS derivatives) [125] in hair.

Deuterated internal standards were commonly used in the cited references, reflecting their wide

Table 6
Summary of published micellar electrokinetic capillary chromatography methods

Reference[a]	Tissue	Drugs	Extraction method	Column/conditions	Derivitisation method	Detection method	Detection limit[b]	Recovery[c]
[126]	5.0 U	Flunitrazepam, oxazepam, diazepam	No hydrolysis, SPE Certify columns, 3-step elution for benzodiazepines and other drugs of abuse	FSC 90 cm×75 μm I.D., 20 kV, various including 75 m*M* SDS, 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4, pH 9.1, and/or organic modifers	None	UV 195–320 nm	100	~80–90
[128]	5–10 U	Numerous (8)	Hydrolysis, SPE Certify columns, 2-step elution	FSC 90–105 cm×75 μm I.D., 20 kV, various including 75 m*M* SDS, 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4, pH 9.2–9.3	None	UV 195–320 nm	n/a	70–90
[127]	2.0 U	Nitrazepam metabolites	No hydrolysis, Sep-Pak C_{18}, DCM elution	FSC 72 cm×50 μm I.D., 20 kV various including 60 m*M* SDS, 6 m*M* phosphate–borate, pH 8.5	None	UV 220 nm	100–200	80–100

n/a – not available or not described.
[a] References in chronological order.
[b] Detection limit in ng/ml.
[c] Recovery in per cent.

commercial availability and their general acceptance as ideal internal standards.

6.3. Micellar electrokinetic capillary chromatography

Micellar electrokinetic capillary chromatography (MECC) has been successfully used to determine benzodiazepines in urine (Table 6) [126–128]. All methods used a sodium dodecyl sulphate–phosphate–borate buffer following solid-phase extraction of the benzodiazepines. Tomita et al. [127] separated nitrazepam and its 7-amino and 7-acetamido metabolites with detection limits of 100–200 ng/ml. Schafroth et al. [128] successfully separated and detected eight benzodiazepines including lorazepam. Wernly and Thormann [126] used solid-phase extraction and MECC to detect drugs of abuse including three benzodiazepines. Multiwavelength UV detection was used to provide an added degree of confirmation [126–128]. Organic modifiers such as methanol (5%), isopropanol (2.5–10%) or acetonitrile (2.5–10%) were shown to assist in the separation process. The sensitivity was shown to be adequate for routine confirmatory analyses of presumptive positive urines for benzodiazepines.

6.4. Thin-layer chromatography

This is the oldest of the chromatographic techniques with a number of textbooks providing detailed information on this procedure. Clarke [15] and Schütz [17,18] are two examples of texts. Traditionally benzodiazepines have been measured as the

Table 7
Benzophenones of selected benzodiazepines

Benzodiazepine	Benzophenone
Bromazepam	(2-Amino-5-bromophenyl)(2-pyridyl)methanone (ABP, ABBP)
Camazepam	2-Methylamino-5-chloro-benzophenone (MACB)
Chlordiazepoxide	2-Amino-5-chlorobenzophenone (ACB)
Clobazam	2-Amino-5-chloro-diphenylamine (ACD)
Clonazepam	2-Amino-2′-chloro-5-nitrobenzophenone (ANCB)
7-Amino-clonazepam	2,5-Diamino-2′-chlorobenzophenone (DACB)
Clorazepate	2-Amino-5-chlorobenzophenone (ACB)
Cloxazolam	2-Amino-5,2′-dichlorobenzophenone (ADB)
Delorazepam	2-Amino-5,2′-dichlorobenzophenone (ADB)
Diazepam	2-Methylamino-5-chloro-benzophenone (MACB)
Fludiazepam	2-Methylamino-5-chloro-2′-fluorobenzophenone (CFMB) 2-Amino-5-chloro-2′-fluorobenzophenone (ACFB)
Flunitrazepam	2′-Fluoro-2-methylamino-5-nitrobenzophenone (MNFB)
Flurazepam	2-Amino-5-chloro-2′-fluorobenzophenone (ACFB)
Haloxazepam	2-Amino-5-bromo-2′-fluorobenzophenone (ABFP)
Ketazolam	2-Methylamino-5-chloro-benzophenone (MACB)
Halazepam	2-(2,2,2-Trifluoroethyl)-amino-5-chlorobenzophenone (TCB)
Lorazepam	2-Amino-2′-5-dichlorobenzophenone (ADB)
Lormetazepam	2′-5-Dichloro-2-(methylamino)benzophenone (MDB)
Medazepam	2-Methylamino-5-chloro-benzophenone (MACB)
Midazolam	2-Amino-5-chloro-2′-fluorobenzophenone (ACFB)
Nimetazepam	2-Methylamino-5-nitrobenzophenone (MNB)
Nitrazepam	2-Amino-5-nitrobenzophenone (ANB)
7-Amino-nitrazepam	2,5-Diaminobenzophenone (DAB)
Nordazepam	2-Amino-5-chlorobenzophenone (ACB)
Oxazepam	2-Amino-5-chlorobenzophenone (ACB)
Oxazolam	2-Amino-5-chlorobenzophenone (ACB)
Pinazepam	5-Chloro-2-(2-propinylamino)benzophenone (CPB)
Prazepam	5-Chloro-2-[(cyclopropylmethyl)amino]benzophenone (CCB, CMCB)
Temazepam	2-Methylamino-5-chloro-benzophenone (MACB)

benzophenones formed after acid treatment of extracts, although alprazolam, loprazolam and triazolam do not form benzophenones (see Table 7 for list of benzophenones) [17,18,129,130]. In the period of this review further developments in the TLC of benzodiazepines have occurred.

Jain [131] used direct TLC-densitometry of benzophenone hydrolysis products of urine extracts of benzodiazepines. The two benzophenones visualised were 2-amino-5-chlorobenzophenone (ACB) and 5-chloro-2-methylaminobenzophenone (MACB), which are formed from nordazepam, oxazepam and diazepam, 3-hydroxydiazepam, respectively. This method used 5 ml of urine and provided a detection limit of 500 ng/ml. This technique avoided the use of visualisation reagents, however in common with other TLC methods specificity was relatively poor.

The use of high-performance TLC plates (HPTLC) has been shown to provide sufficient discrimination power to detect 16 benzodiazepines using 3 solvent systems [132]. The detection limits ranged from 100–1000 ng/ml allowing benzodiazepines to be detected in 1 ml of serum in suspected poisoning cases.

In a further development HPTLC was used to detect flunitrazepam and its metabolites by the hydrolysis and subsequent formation of acridine derivatives by cyclization in dimethyl formamide [133]. The use of alternative solvent systems allowed discrimination from diclofenac, carbamazepine and tricyclic antidepressants. The colour of a large number of benzodiazepines have been more recently described on this system [134].

6.5. Chiral separations

Baseline enantiomeric separation of eight racemic benzodiazepines has been described on HPLC using two types of substituted methylphenylcarbamates on cellulose columns [135]. Mawa et al. [98,136] used HPLC to separate the stereoisomers of oxazepam glucuronides from oxazepam using a conventional C_{18} reversed-phase column and an acetonitrile–2-propanol–phosphoric acid mobile phase. It should be noted that the two enantiomeric forms of oxazepam racemise in solution preventing useful enantiomeric separation of oxazepam. This process is probably common to the 3-hydroxy benzodiazepines.

7. Conclusions

A number of commercial screening tests are now available to provide an initial test for the presence of benzodiazepines in specimens. Their applicability will depend on the tissue being examined (i.e. blood/plasma/serum or urine) and the type of benzodiazepines likely to be present in the specimen. Prior enzymatic hydrolysis to convert glucuronide metabolites to immunoreactive species is most favoured to improve the detectability. The use of lower (than recommended) cut-off limits in immunoassays offers an improved degree of detectability particularly for the more potent benzodiazepines such as triazolam and lorazepam. The combination of the two techniques is recommended for optimum sensitivity. However, individual cross-reactivities of immunoassays will always need to be considered in any on-site use of such tests. Interestingly, radio receptor assays are becoming increasingly accepted as an alternative to immunoassays and offer the distinct advantage that detectability is not drug dependent.

Again there are a number confirmation tests available for benzodiazepines. Most favoured in terms of number of publications in the time period under review were HPLC techniques, although both GC and GC–MS techniques were also strongly represented. HPLC techniques were capable of detecting most of the benzodiazepines using UV absorbance detection, although DAD detection provided an improved means to exclude coeluting substances. There was no obvious preference for choice of column, except reversed-phase C_{18} columns which were more prevalent than the alternative phases. Isolation steps were almost evenly distributed between conventional liquid–liquid extraction and solid-phase extraction procedures. There was no obvious advantage in choice of isolation procedure for the whole class of benzodiazepines. Interestingly, the combination of HPLC with MS appears to offer an important alternative to conventional GC–MS.

Conventional GC methods using fused capillary columns were most commonly used with ECD detection, although some papers found NPD detection a useful alternative detector. Slightly polar phases from 5% diphenyldimethylsiloxane phases to more polar phases were preferred for best separation capability. Possibly because of the detector type,

none of these methods derivatised the benzodiazepines.

GC–MS methods were, as expected, well represented and still offer the best means to confirm benzodiazepines. Most of these methods derivatised the benzodiazepine to both improve spectral definition and to reduce thermal degradation on-column. Derivatives most favoured were the conventional trimethylsilyl, although the *t*-butyldimethylsilyl derivative provided offered improved spectral properties. Isolation steps again showed wide variability from conventional liquid–liquid phase to solid-phase methods with no obvious relative advantage. Dimethylpolysiloxane (100%) and 5% diphenyldimethylpolysiloxane phases were most commonly used and provided good separation capabilities for most derivatised benzodiazepines. Ionisation methods were generally EI with selected ion monitoring, although the use of NCI gave significant improvements in sensitivity with detection limits down to 0.1 ng/ml.

Other techniques are described. Micellar electrokinetic chromatography has been shown to be capable of the analysis of benzodiazepines in urine. TLC has shown little interest in the scientific publications over the last 5 years. This technique still suffers from a lack of sensitivity and specificity, particularly for the newer more potent benzodiazepines.

Acknowledgements

The assistance of Kerrie Johannes as resourceful librarian is gratefully acknowledged.

References

[1] In: Recommended methods for the detection and assay of barbiturates and benzodiazepines in biological specimens, United Nations International Drug Control Programme publication ST/NAR/28, 1997, Vienna.
[2] J.H. Woods, J.L. Katz, G. Winger, Pharmacol. Rev. 39 (1987) 254–390.
[3] R.T. Owen, P. Tyrer, Drugs 25 (1983) 385–398.
[4] O.H. Drummer, D.L. Ranson, Am. J. Forensic Med. Pathol. 17 (1996) 336–342.
[5] K. Worm, A. Steentoft, J. Toft, J. Traffic Med. 24 (1996) 39–42.
[6] K. Worm, A. Steentoft, H. Christensen, Traffic Med. 19 (1991) 3–8.
[7] O.H. Drummer, D.L. Ranson, Am. J. Forensic Med. Pathol. 17 (1996) 336–342.
[8] I.M. McIntyre, M.L. Syrjanen, K. Crump, S. Horomidis, A.W. Peace, O.H. Drummer, J. Anal. Toxicol. 17 (1993) 202–207.
[9] S.D. Ferrara, L. Tedeschi, G. Frison, F. Castagna, J. Anal. Toxicol. 16 (1992) 217–222.
[10] W. Huang, D.E. Moody, J. Anal. Toxicol. 19 (1995) 333–342.
[11] H.H. Maurer, J. Chromatogr. B 580 (1992) 3–41.
[12] H. Maurer, K. Pfleger, J. Chromatogr. 422 (1987) 85–101.
[13] R.L. Hagan, J. Anal. Toxicol. 19 (1995) 58.
[14] R.L. Hagan, California Association of Toxicologists Newsletter, 1995, pp. 22–33.
[15] Clarke's Isolation and Identification of Drugs, 2nd edition, The Pharmaceutical Press, London, 1986.
[16] A.D. Fraser, A.F. Isner, W. Bryan, J. Anal. Toxicol. 17 (1993) 427–431.
[17] H. Schütz (Ed.), Basic data, analytical methods, pharmacokinetics, and comprehensive literature, In: Benzodiazepines – A Handbook, Vol. 1, Springer Verlag, Heidelberg, 1982.
[18] H. Schütz (Ed.), Basic data, analytical methods, pharmacokinetics, and comprehensive literature, In: Benzodiazepines – A Handbook, Vol. 2, Springer Verlag, Heidelberg, 1982.
[19] R.C. Baselt (Ed.), In: Advances in Analytical Toxicology 2, Biomedical Publishers, Foster City, CA, USA, 1989, Chap. 1.
[20] Jones, Abused drugs monograph series, H. Caplan (Ed.), Benzodiazepines, Abbott Laboratories, 1994.
[21] M.D. Robertson, O.H. Drummer, J. Forensic Sci. 40 (1995) 382–386.
[22] R. Meatherall, J. Anal. Toxicol. 18 (1994) 385–390.
[23] R. Meatherall, J. Anal. Toxicol. 18 (1994) 382–384.
[24] L.E. Edinboro, A. Poklis, J. Anal. Toxicol. 18 (1994) 312–316.
[25] R.E. West, D.P. Ritz, J. Anal. Toxicol. 17 (1993) 114–116.
[26] W.A. Joern, J. Anal. Toxicol. 16 (1992) 363–367.
[27] P.H. Dickson, W. Markus, J. McKernan, H.C. Nipper, J. Anal. Toxicol. 16 (1992) 67–71.
[28] R.L. Fitzgerald, D.A. Rexin, D.A. Herold, J. Anal. Toxicol. 17 (1993) 342–347.
[29] C. Moore, G. Long, M. Marr, J. Chromatogr. B 655 (1994) 132–137.
[30] J.L. Valentine, R. Middleton, C. Sparks, J. Anal. Toxicol. 20 (1996) 416–424.
[31] P. Simonsson, A. Lidén, S. Lindberg, Clin. Chem. 41 (1995) 920–923.
[32] R.L. Fitzgerald, D.A. Rexin, D.A. Herold, Clin. Chem. 40 (1994) 373–380.
[33] A.D. Fraser, R. Meatherall, J. Anal. Toxicol. 20 (1996) 217–223.

[34] T.R. Koch, R.L. Raglin, S. Kirk, J.F. Bruni, J. Anal. Toxicol. 18 (1994) 168–172.
[35] O. Beck, P. Lafolie, P. Hjemdahl, S. Borg, G. Odelius, P. Wirbing, Clin. Chem. 38 (1992) 271–275.
[36] R.L. Fitzgerald, S. Priem, T. Leech, K. Schlusse, D.A. Herold, Clin. Chem. 41 (1995) S125.
[37] D. Laurie, A.J. Mason, N.H. Piggott, F.J. Rowell, J. Saviour, D. Strachan, J.D. Tyson, Analyst 121 (1996) 951–954.
[38] A.D. Fraser, W. Bryan, J. Anal. Toxicol. 19 (1995) 281–284.
[39] A.D. Fraser, W. Bryan, J. Anal. Toxicol. 15 (1991) 63–65.
[40] A.D. Fraser, W. Bryan, A.F. Isner, J. Anal. Toxicol. 16 (1992) 347–350.
[41] A.D. Fraser, W. Bryan, A.F. Isner, J. Anal. Toxicol. 15 (1991) 8–12.
[42] N. Bellet, J. Anal. Toxicol. 21 (1997) 76.
[43] J. Becker, A. Correll, W. Koepf, C. Rittner, J. Anal. Toxicol. 17 (1993) 103–108.
[44] W. Huang, D.E. Moody, D.M. Andrenyak, D.E. Rollins, J. Anal. Toxicol. 17 (1993) 365–369.
[45] P.G. Agbuya, L. Li, M.V. Miles, A.L. Zaritsky, A.D. Morris, Ther. Drug Monit. 18 (1996) 194–199.
[46] P.D. Camara, L. Audette, K. Velletri, P. Breitenbecher, M. Roaner, W.C. Griffiths, Clin. Chem. 41 (1995) 115.
[47] T. Matuch-Hite, P. Jones, J. Moriarty, J. Anal. Toxicol. 19 (1995) 130.
[48] A.H. Wu, E. Forte, G. Casella, K. Sun, G. Hemphill, R. Foery, H. Schanzenbach, J. Forensic Sci. 40 (1995) 614–618.
[49] R.L. Fitzgerald, D.A. Herold, J. Anal. Toxicol. 21 (1997) 32–35.
[50] N. Bellet, H.A. Scholz, M.L. Opel, E. Padilla, F.G. Roark, P. Khanna, J. Anal. Toxicol. 20 (1996) 76.
[51] A. West, H. Kohler-Schmidt, S. Baudner, G. Blaschke, Int. J. Legal Med. 108 (1995) 105–109.
[52] J. Bruhwyler, A. Hassoun, J. Anal. Toxicol. 17 (1993) 403–407.
[53] B. Borggaard, I. Joergensen, J. Anal. Toxicol. 18 (1994) 243–246.
[54] T. Nishikawa, S. Suzuki, H. Ohtani, N.W. Eizawa, T. Sugiyama, T. Kawaguchi, S. Miura, Am. J. Clin. Pathol. 102 (1994) 605–610.
[55] K. Ensing, I.J. Bosman, A.C.G. Egberts, J.P. Franke, R.A. De Zeeuw, J. Pharm. Biomed. Anal. 12 (1994) 53–58.
[56] T. Nishikawa, H. Ohtani, D.A. Herold, R.L. Fitzgerald, Am. J. Clin. Pathol. 107 (1997) 345–352.
[57] K. Ensing, I.J. Bosman, A.C.G. Egberts, J.P. Franke, R.A. De Zeeuw, J. Pharm. Biomed. Anal. 12 (1994) 59–63.
[58] P. Kintz, P. Mangin, J. Anal. Toxicol. 15 (1991) 202–203.
[59] A.M.A. Verweij, P.J.L. Lipman, P.G.M. Zweipfenning, Forensic Sci. Int. 54 (1992) 67–74.
[60] H.A. Adams, B. Weber, B. Bachmann-M, M. Gúerin, G. Hempelmann, Anaesthetist 41 (1992) 619–624.
[61] H. Le Solleu, F. Demotes-Mainard, G. Vincon, B. Bannwarth, J. Pharm. Biomed. Anal. 11 (1993) 771–775.
[62] K. Chan, R.D.M. Jones, J. Chromatogr. 619 (1993) 154–160.
[63] L. Jin, C.E. Lau, J. Chromatogr. B 654 (1994) 77–83.
[64] F. Benhamou-Batut, F. Demotes-Mainard, L. Labat, G. Vincon, B. Bannwarth, J. Pharm. Biomed. Anal. 12 (1994) 931–936.
[65] B. Lehmann, R. Boulieu, J. Chromatogr. B 674 (1995) 138–142.
[66] R. Mariot, L. Zangirolami, J. Chromatogr. B 677 (1996) 190–193.
[67] P. Bouget, V. Bouton, A. Lesne-Hulin, P. Amstutz, M. Benayed, D. Benhamou, P.L. Dufieux, G. Goursot, S. Grosbuis, J.P. Haberer, F. Jardin, P. Kirstetter, J. Marty, A. Mercatello, B. Page, J.L. Pourriat, T. Vassal, Ther. Drug Monit. 18 (1996) 610–619.
[68] D.F. LeGatt, D.P. McIntosh, Clin. Biochem. 26 (1993) 159–163.
[69] R. Nishioka, J. Chromatogr. B 681 (1996) 401–404.
[70] W. Rieck, D. Platt, J. Chromatogr. B 578 (1992) 259–263.
[71] K. Chiba, H. Horii, T. Chiba, Y. Kato, T. Hirano, T. Ishizaki, J. Chromatogr. B 668 (1995) 77–84.
[72] M.D. Robertson, O.H. Drummer, J. Chromatogr. B 667 (1995) 179–184.
[73] O.H. Drummer, S. Horomidis, S. Kourtis, M.L. Syrjanen, P. Tippett, J. Anal. Toxicol. 18 (1994) 134–138.
[74] T.V. Beischlag, T. Inaba, J. Anal. Toxicol. 16 (1992) 236–239.
[75] T. Edeki, D.W. Robin, C. Prakash, I.A. Blair, A.J.J. Wood, J. Chromatogr. 577 (1992) 190–194.
[76] J.F. Kelly, D.J. Greenblatt, J. Chromatogr. 621 (1993) 102–104.
[77] L.E. Fisher, S. Perch, M.F. Bonfiglio, S.M. Geers, J. Chromatogr. B 665 (1995) 217–221.
[78] H. Gjerde, E. Dahlin, A.S. Christophersen, J. Pharm. Biomed. Anal. 10 (1992) 317–322.
[79] K. Wolff, D. Garretty, A.W.H. Hay, Ann. Clin. Biochem. 34 (1997) 61–67.
[80] J.M. Duthel, H. Constant, J.J. Vallon, T. Rochet, S. Miachon, J. Chromatogr. 579 (1992) 85–91.
[81] D.M. Song, S. Zhang, K. Kohlhof, J. Chromatogr. B 658 (1994) 142–148.
[82] D.M. Song, S. Zhang, K. Kohlhof, J. Chromatogr. B 660 (1994) 95–101.
[83] D.M. Song, V. Khaykis, K. Kohlhof, J. Chromatogr. B 663 (1995) 263–273.
[84] D.M. Song, S. Zhang, K. Kohlhof, J. Chromatogr. B 686 (1996) 199–204.
[85] S.B. Needleman, M. Porvaznik, Forensic Sci. Int. 73 (1995) 49–60.
[86] B.-I. Podkowik, S. Masur, J. Chromatogr. B 681 (1996) 405–411.
[87] F. Berthault, P. Kintz, P. Mangin, J. Chromatogr. 685 (1996) 383–387.
[88] F. Mußhoff, T. Daldrup, Int. J. Legal Med. 105 (1992) 105–109.
[89] H.-L. Tai, R.J. Osiewicz, D.P. Bofinger, Clin. Biochem. 26 (1993) 165–171.
[90] M. Casas, L.A. Berrueta, B. Gallo, F. Vicente, J. Pharm. Biomed. Anal. 11 (1993) 277–284.

[91] F. Kamali, J. Pharm. Biomed. Anal. 11 (1993) 625–627.
[92] J. Guichard, G. Panteix, J. Dubost, P. Baltassat, C. Roche, J. Chromatogr. 612 (1993) 269–275.
[93] J. Chopineau, F. Rivault, V. Sautou, M.F. Sommier, J. Liq. Chromatogr. 17 (1994) 373–383.
[94] B.C. Sallustio, C. Kassapidis, R.G. Morris, Ther. Drug Monit. 16 (1994) 174–178.
[95] J. Guitton, D. Durand, D. Bouillod, M. Manchon, Ann. Biol. Clin. 52 (1994) 111–115.
[96] K.K. Åkerman, J. Jolkkonen, M. Parviainen, I. Penttilä, Clin. Chem. 42 (1996) 1412–1416.
[97] M. Kleinschmidt, M. Herderich, P. Schreier, J. Chromatogr. B 676 (1996) 61–67.
[98] R. Mawa, D. Mis, M.C. Cagnieu, D. Grancher, M. Petit-Ramel, F. Bressolle, J.-J. Vallon, J. Chromatogr. B 677 (1996) 331–338.
[99] K.K. Åkerman, Scand. J. Clin. Lab. Invest. 56 (1996) 609–614.
[100] L.A. Berrueta, B. Gallo, F. Vicente, J. Chromatogr. 616 (1993) 344–348.
[101] T. Kondo, D.C. Buss, P.A. Routledge, Ther. Drug Monit. 15 (1993) 35–38.
[102] Z. Lin, O. Beck, J. Pharm. Biomed. Anal. 13 (1995) 719–722.
[103] L. Deinl, L. von Meyer, Proceedings of TIAFT Meeting, Leipzig, 1993, pp. 171–177.
[104] Z.-P. Huang, X.-H. Chen, J. Wijsbeek, J.-P. Franke, R.A. De Zeeuw, J. Anal. Toxicol. 20 (1996) 248–254.
[105] D.A. Black, G.D. Clark, V.M. Haver, J.A. Garbin, A.J. Saxon, J. Anal. Toxicol. 18 (1994) 185–188.
[106] A.M.A. Verweij, M.L. Hordijk, P.J.L. Lipman, J. Chromatogr. B 686 (1996) 27–34.
[107] X.-H. Chen, J.-P. Franke, K. Ensing, J. Wijsbeek, R.A. De Zeeuw, J. Anal. Toxicol. 17 (1993) 421–426.
[108] C. Lacroix, F. Wojciechowski, P. Danger, J. Chromatogr. 617 (1993) 285–290.
[109] R. Lauber, M. Mosimann, M. Bührer, A.M. Zbinden, J. Chromatogr. B 654 (1994) 69–75.
[110] H. Iwase, K. Gondo, T. Koike, I. Ono, J. Chromatogr. B 655 (1994) 73–81.
[111] N.C. Van der Merbel, J.M. Teule, H. Lingeman, U.A.Th. Brinkman, J. Pharm. Biomed. Anal. 10 (1992) 225–233.
[112] M.A. Hall, C.A. Robinson, R.M. Brissie, J. Anal. Toxicol. 19 (1995) 511–513.
[113] H. Essien, S.J. Lai, S.R. Binder, D.L. King, J. Chromatogr. B 683 (1996) 199–208.
[114] V. Mastey, A.-C. Panneton, F. Donati, F. Varin, J. Chromatogr. B 655 (1994) 305–310.
[115] O.H. Drummer, A. Kotsos, I.M. McIntyre, J. Anal. Toxicol. 17 (1993) 225–229.
[116] W.E. Lambert, J.F. Van Bocxlaer, A.P. De Leenheer, J. Chromatogr. B 689 (1997) 45–53.
[117] P. Demedts, A. Wauters, F. Franck, H. Neels, Eur. J. Clin. Chem. Clin. Biochem. 32 (1994) 409–417.
[118] R. Herraez-Hernandez, A.J.H. Louter, N.C. Van der Merbel, J. Pharm. Biomed. Anal. 14 (1996) 1077–1087.
[119] M. Krogh, H. Grefslie, K.E. Rasmussen, J. Chromatogr. B 689 (1997) 357–364.
[120] R. Meatherall, J. Anal. Toxicol. 18 (1994) 369–381.
[121] J.R. Joyce, J.T.S. Bal, R.E. Andrey, H.M. Stevens, A.C. Moffat, Biomed. Mass Spectrom. 11 (1984) 284–289.
[122] J.D. McCarley, J. Brodbelt, Anal. Chem. 65 (1993) 2380–2388.
[123] V. Cirimele, P. Kintz, P. Mangin, J. Anal. Toxicol. 20 (1996) 596–598.
[124] F. Vayssette, V. Cirimele, P. Kintz, P. Mangin, Ann. Pharm. Franc. 54 (1996) 211–216.
[125] K.M. Höld, D.J. Crouch, D.G. Wilkins, D.E. Rollins, D.V. Canfield, J. Anal. Toxicol. 20 (1996) 65.
[126] P. Wernly, W. Thormann, Anal. Chem. 64 (1992) 2155–2159.
[127] M. Tomita, T. Okuyama, S. Sato, H. Ishizu, J. Chromatogr. 621 (1993) 249–255.
[128] M. Schafroth, W. Thormann, D. Allemann, Electrophoresis 15 (1994) 72–78.
[129] H. Schütz, TLC data of hydrolysis products of 1,4- and 1,5-benzodiazepines, J. Anal. Toxicol. 2 (1978) 147–148.
[130] In: TLC R_f values of toxicologically relevant substances on stanardized systems, Report XVII of the DFG Commission for Clinical–Toxicological Analysis, Special Issue of the TIAFT Bulletin, VCH, 1992.
[131] R. Jain, J. Chromatogr. 615 (1993) 365–368.
[132] K. Otsubo, H. Seto, K. Futagami, R. Oishi, J. Chromatogr. B 669 (1995) 408–412.
[133] G. Rochholz, B. Ahrens, H. Schütz, Arzneim.-Forsch. 44 (1994) 469–471.
[134] G. Rochholz, A. Mayr, H. Schütz, Fresensius J. Anal. Chem. 346 (1993) 819–827.
[135] B. Chankvetadze, L. Chankvetadze, Sh. Sidamonidze, E. Yashima, Y. Okamoto, J. Pharm. Biomed. Anal. 14 (1996) 1295–1303.
[136] R.V. Mawa, J.J. Vallon, M.C. Cagnieu, M. Petit-Ramel, D. Grancher, Anal. Lett. 28 (1995) 1387–1404.