Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 2/3, pp. 109–136, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

Review

Biopharmacological data and high-performance liquid chromatographic analysis of 1,4-benzodiazepines in biological fluids: a review

L.A. BERRUETA,* B. GALLO and F. VICENTE

Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo, 644-E, 48080 Bilbao, Spain

Abstract: A review with 123 references on the analysis of 1,4-benzodiazepines in biological samples using HPLC is presented. Some important physico-chemical and biopharmacological data for the development of analytical methods are collected. Different methods of sample pretreatment, chromatographic conditions and detection systems are discussed.

Keywords: *1,4-Benzodiazepines; biopharmacological data; HPLC; analysis in biological fluids.*

Introduction

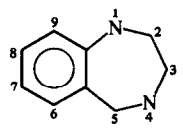
Since the introduction of chlordiazepoxide (Librium™) in 1960, a large number of other 1,4-benzodiazepines have been investigated for biological activity. Due to their tranquilizer, anti-depressive and sedative properties they have become the most used medicines in the treatment of anxiety and sleep disturbances.

In recent years a large number of analytical and pharmacological studies on 1,4-benzodiazepines have been described. For the biopharmacological, clinical and toxicological studies of these drugs the availability of rapid, sensitive and selective analytical methods for their determination in biological fluids is essential.

This article aims to review the literature that has appeared in the last few years on the analysis of 1,4-benzodiazepines in biological fluids using HPLC. Also, it will pay attention to the physico-chemical and biological data and properties of these drugs that can be useful in the development of suitable analytical methods. Some previous reviews on this subject have been published [1–3]. Two books that contain some analytical data pertaining to 1,4-benzodiazepines have been edited by Schütz [4, 5].

Physico-chemical Properties

The main chemical characteristic of 1,4-benzodiazepines is the presence of a benzene ring fused to a saturated seven membered ring with nitrogens at the 1 and 4 positions.



The most important types of 1,4-benzodiazepines according to their chemical structure are the following:

(a) 2-keto derivatives, characterized by the presence of a C=O group at position 2 (Fig. 1, structures A and C);

(b) 4-*N*-oxide derivatives, with a *N*-oxide group at position 4 (Fig. 1, structures B and C);

(c) triazolo derivatives, characterized by the presence of an additional five membered ring with two nitrogen atoms at positions 3 and 4 relative to the nitrogen at position 1 (Fig. 1, structure D);

(d) imidazo derivatives, with an additional five membered ring but with only one nitrogen atom (Fig. 1, structure E);

(e) oxazolo derivatives, with the presence of an additional five membered ring with an oxygen atom (Fig. 1, structure F).

Other 1,4-benzodiazepines not included in these major groups are also presented in Fig. 1 (structures G–J). Names and substituents of the most important 1,4-benzodiazepine drugs are shown in Table 1.

* Author to whom correspondence should be addressed.

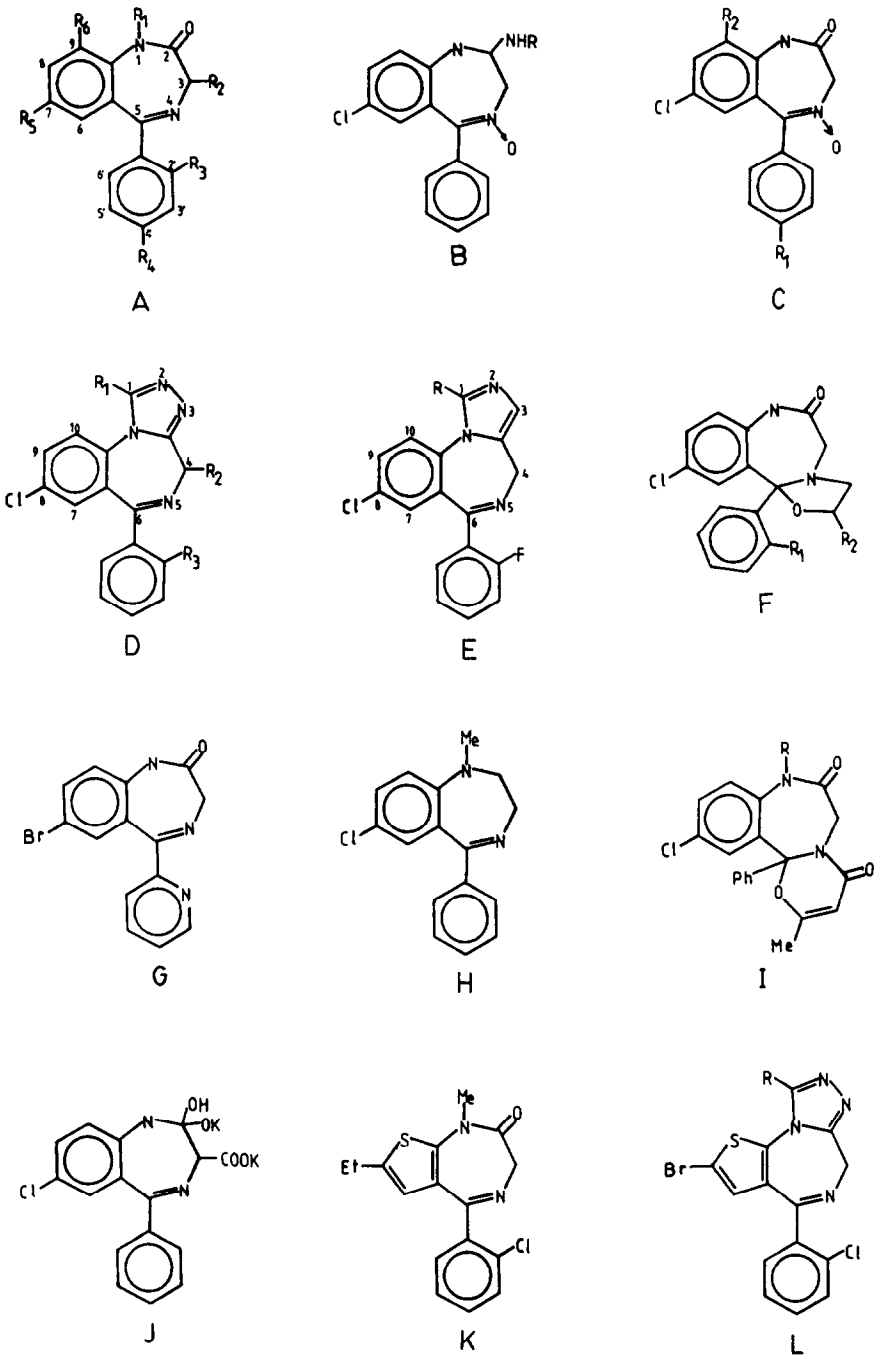


Figure 1
Chemical structures of some 1,4-benzodiazepines and related compounds. B (R = —CH_3): Chlordiazepoxide. B (R = —H): Desmethylchlordiazepoxide. E (R = —CH_3): Midazolam. E (R = —CH_2OH): 1-Hydroxymethylmidazolam. G: Bromazepam. H: Medazepam. I (R = —CH_3): Ketazolam. I (R = —H): Desmethylketazolam. J: Clorazepate. K: Clotiazepam. L (R = —CH_3): Brotizolam. L (R = —cyclohexyl): Cyclotizolam.

Also, 1,4-benzodiazepine analogues have been synthesized, such as thienodiazepines (Clotiazepam) or thienotriazolodiazepines (Brotizolam, Ciclotizolam). In these compounds the basic chemical structure of 1,4-benzodiazepines has been modified as illustrated in Fig. 1, structures K and L.

Data on the synthesis of some 1,4-benzodiazepines can be found in the publications of Seiler and Zimmermann [6], Hümpel *et al.* [7] and Eberts *et al.* [8].

1,4-Benzodiazepines are basic in character and as neutral molecules are soluble in organic solvents such as methanol, ethanol, dimethyl-

Table 1
Names and substituents of some 1,4-benzodiazepines with relation to the structures shown in Fig. 1

Name	Structure A					
	R_1	R_2	R_3	R_4	R_5	R_6
Diazepam	$-CH_3$	—H	—H	—H	—Cl	—H
Temazepam*	$-CH_3$	—OH	—H	—H	—Cl	—H
Oxazepam*	—H	—OH	—H	—H	—Cl	—H
Nordazepam*	—H	—H	—H	—H	—Cl	—H
Flunitrazepam	$-CH_3$	—H	—F	—H	$-NO_2$	—H
7-Aminoflunitrazepam†	$-CH_3$	—H	—F	—H	$-NH_2$	—H
7-Acetamidoflunitrazepam†	$-CH_3$	—H	—F	—H	$-NHCOCH_3$	—H
Norflunitrazepam†	—H	—H	—F	—H	$-NO_2$	—H
3-Hydroxyflunitrazepam†	$-CH_3$	—OH	—F	—H	$-NO_2$	—H
7-Amino-norflunitrazepam†	—H	—H	—F	—H	$-NH_2$	—H
Lormetazepam	$-CH_3$	—OH	—Cl	—H	—Cl	—H
Lorazepam*	—H	—OH	—Cl	—H	—Cl	—H
Delorazepam*	—H	—H	—Cl	—H	—Cl	—H
Flurazepam	$-(CH_2)_2NEt_2$	—H	—F	—H	—Cl	—H
N-1-Desalkylflurazepam†	—H	—H	—F	—H	—Cl	—H
Monodesethylflurazepam†	$-(CH_2)_2NHEt$	—H	—F	—H	—Cl	—H
Didesethylflurazepam†	$-(CH_2)_2NH_2$	—H	—F	—H	—Cl	—H
N-1-Hydroxyethylflurazepam†	$-CH_2CH_2OH$	—H	—F	—H	—Cl	—H
N-1-Desalkyl-3-hydroxyflurazepam‡	—H	—OH	—F	—H	—Cl	—H
N-1-Flurazepam acetic acid‡	$-CH_2COOH$	—H	—F	—H	—Cl	—H
Clonazepam	—H	—H	—Cl	—H	$-NO_2$	—H
3-Hydroxyclonazepam†	—H	—OH	—Cl	—H	$-NO_2$	—H
7-Aminoclonazepam†	—H	—H	—Cl	—H	$-NH_2$	—H
7-Amino-3-hydroxyclonazepam†	—H	—OH	—Cl	—H	$-NH_2$	—H
7-Acetamidoclonazepam†	—H	—H	—Cl	—H	$-NHCOCH_3$	—H
7-Acetamido-3-hydroxyclonazepam†	—H	—OH	—Cl	—H	$-NHCOCH_3$	—H
Nitrazepam	—H	—H	—H	—H	$-NO_2$	—H
7-Aminonitrazepam†	—H	—H	—H	—H	$-NH_2$	—H
7-Acetamidonitrazepam†	—H	—H	—H	—H	$-NHCOCH_3$	—H
Prazepam	$-CH_2-\triangleleft$	—H	—H	—H	—Cl	—H
Fludiazepam	$-CH_3$	—H	—F	—H	—Cl	—H
Nimetazepam	$-CH_3$	—H	—H	—H	$-NO_2$	—H
Camazepam	$-CH_3$	$-OCON(CH_3)_2$	—H	—H	—Cl	—H
N-Desoxy-5-(4-hydroxyphenyl)demoxepam§	—H	—H	—H	—OH	—Cl	—H
N-Desoxy-9-hydroxydemoxepam§	—H	—H	—H	—H	—Cl	—OH
Pinazepam	$-CH_2-C\equiv CH$	—H	—H	—H	—Cl	—H

Table 1
Continued

Structure C			
Name	R_1	R_2	
Demoxepam	—H	—H	
5-(4-Hydroxyphenyl)demoxepam†	—OH	—H	
9-Hydroxydemoxepam†	—H	—OH	
Structure F			
Name	R_1	R_2	
Cloxazolam	—Cl	—H	
Oxazolam	—H	$—CH_3$	
Haloxazolam	—F	—H	
Structure D			
Name	R_1	R_2	R_3
Triazolam	$—CH_3$	—H	—Cl
4-Hydroxytriazolam†	$—CH_3$	—OH	—Cl
1-Hydroxymethyltriazolam†	$—CH_2OH$	—H	—Cl
4-Hydroxy-1-hydroxymethyltriazolam†	$—CH_2OH$	—OH	—Cl
Alprazolam	$—CH_3$	—H	—H
Estazolam	—H	—H	—H

Metabolites are collected below the parent 1,4-benzodiazepine.
*Metabolites of some active 1,4-benzodiazepines and also active compounds used in formulations.
†Metabolites of some active 1,4-benzodiazepines.
‡Metabolites detected in dog.
§Metabolites of Demoxepam.

formamide and chloroform, but only slightly soluble in *n*-hexane or *n*-heptane and practically insoluble in water. In contrast, the salt forms (e.g. Chlordiazepoxide and Flurazepam hydrochlorides, Loprazolam methanesulphonate, dipotassium clorazepate) are water soluble. Data on the water solubility of 1,4-benzodiazepines can be found in the publications of Carstensen *et al.* [9] and Windholz and Budavari [10]. Despite their very low solubility in water, 1,4-benzodiazepines are found to be totally soluble in the concentration ranges at which they appear in bioanalytical samples [11]. Thus, aqueous solubility of Diazepam is 50 $\mu g\ ml^{-1}$ [12]; for Clonazepam solubility is highest at pH = 2.0 (76 $\mu g\ ml^{-1}$) and lowest at pH = 7.4 (19 $\mu g\ ml^{-1}$); and for Flunitrazepam aqueous solubility decreases when pH increases from 850 $\mu g\ ml^{-1}$ at pH = 1 to 11 $\mu g\ ml^{-1}$ at pH = 5.3 [13].

Stock solutions of 1,4-benzodiazepines in methanol and ethanol are stable for several weeks with the exception of Medazepam [1, 14]. Thus, 1000 $\mu g\ ml^{-1}$ solutions of Alprazolam, Triazolam, Lorazepam and Hydroxyalprazolam in methanol are stable for 10 months when they are stored in the dark at −15°C [15]. 1000 $\mu g\ ml^{-1}$ solutions of Diazepam, Oxazepam, Nordazepam and Temazepam in 95% ethanol are stable under refrigeration for several months [14]. Stock solutions of Clobazam and Desmethylclobazam in methanol are reported to be stable for several weeks at 4°C [16]. However, *N*-desmethylchlordiazepoxide solutions in methanol are found to be unstable when stored at 4°C in the dark [17].

In aqueous or aqueous-alcoholic solution, most 1,4-benzodiazepines undergo hydrolysis, particularly under acidic or alkaline conditions [11]. Depending upon the different conditions and the 1,4-benzodiazepine type, hydrolysis can affect the 3,4-azomethine group, the 1,2-amidic bonds, or both, producing the corresponding benzophenone.

The hydrolysis processes have been studied by several authors and the resulting benzophenones have been used for the detection and determination of 1,4-benzodiazepines [11, 18, 19]. In these methods the breakdown of different 1,4-benzodiazepines to the same benzophenone would seem to be a disadvantage, however in certain circumstances it could be an advantage because a particular 1,4-benzodiazepine and its metabolites can yield the same benzophenone, thus increasing sensitivity. Also, in some cases (Nitrazepam, Flunitrazepam and Clonazepam) two or three different benzophenones can be formed [11].

The acid–base characteristics of 1,4-benzodiazepines are due to the nitrogen atom in position 4 which can be protonated, except in 4-*N*-oxide-derivatives. Other nitrogen atoms, as in the 7-amino derivatives, also can be protonated. The hydroxyl group in the 3-hydroxy derivatives can be deprotonated at high pH values whilst the *N*-oxide group in 4-*N*-oxide-derivatives is protonated at low pH values. pK_a values and the acid–base species proposed for some 1,4-benzodiazepines are given in Table 2. Acid–base properties and pK_a values of some 1,4-benzodiazepines in the Hammett acidity region have also been reported [26].

Some physical and chemical properties of particular 1,4-benzodiazepines can be found in the publications of Windholz and Budavari [10] and Florey [27]. Seiler and Zimmermann [6] have identified a number of relationships between physico-chemical and structural parameters. Other relationships between structure and electrochemical properties [28], and between structure and pharmacological activity [29] have also been studied.

Metabolism and Pharmacokinetic Data

Analysis of 1,4-benzodiazepines in biological fluids requires a previous knowledge of the biotransformations that they can undergo

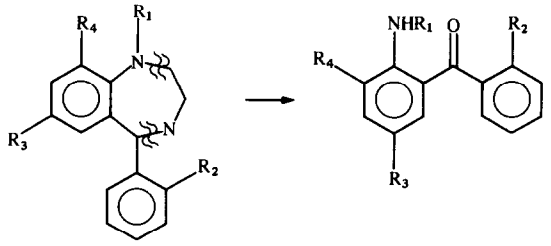


Table 2
pK_a values for some 1,4-benzodiazepines

Name	H_3A^{2+}	pK_a	H_2A^+	pK_a	HA	pK_a	A^-	Ref.
7-Acetamidonitrazepam		—	^{4}N—H	3.2	*	12.4	^{3}C=^{2}C—O$^-$	20
7-Aminoflunitrazepam		—	^{4}N—H	3.55	*	—		6
7-Aminonitrazepam		—	^{4}N—H	4.51	*	12.98	^{1}N	6
	$^7NH_3$	2.5	^{4}N—H	4.6	*	13.1	^{3}C=^{2}C—O$^-$	20
Chlordiazepoxide		—	^{4}N—OH	4.6	*	—		21
Chlordiazepoxide lactam		—	^{4}N—OH	4–5	*	11.5	^{3}C=^{2}C—O$^-$	20
Clonazepam		—	^{4}N—H	1.57	*	10.50	^{1}N	6
		—	^{4}N—H	1.5	*	10.5	^{1}N	13
Desalkylflurazepam		—	^{4}N—H	2.57	*	11.77	^{1}N	6
Delorazepam		—	^{4}N—H	2.17	*	11.80	^{1}N	6
Demoxepam		—		—	*	10.7		22
Diazepam		—	^{4}N—H	3.3	*	—		21
		—		3.4		—		10
		—	^{4}N—H	3.17	*	—		6
		—		3.4	*	—		23
Fludiazepam		—	^{4}N—H	2.22	*	—		6
Flunitrazepam		—	^{4}N—H	1.87	*	—		6
		—	^{4}N—H	1.8	*	—		13
		—	^{4}N—H	1.86	*	—		24
Flurazepam		—	^{4}N—H	1.4	*	—		1
Lorazepam		—	^{4}N—H	1.3	*	11.5	^{3}C—O$^-$	1,21
		—		1.3		11.5		1
		—	^{4}N—H	0.39	*	11.03	^{1}N	6
		—	^{4}N—H	0.96	*	11.15	^{3}C—O$^-$	25
Medazepam		—	^{4}N—H	4.4	*	—		21
Nimetazepam		—	^{4}N—H	2.63	*	—		6
Nitrazepam		—	^{4}N—H	2.88	*	10.88	^{1}N	6
		—	^{4}N—H	3.2	*	10.8	^{1}N=^{2}C—O$^-$	21
Nordazepam		—	^{4}N—H	3.48	*	11.82	^{1}N	6
		—	^{4}N—H	3.5	*	12.0	^{3}C=^{2}C—O$^-$	20
Oxazepam		—	^{4}N—H	1.62	*	11.24	^{1}N	6
		—	^{4}N—H	1.7	*	11.6	^{3}C—O$^-$	21

*Neutral molecule.

whilst in an organism, a subject that has been reviewed by Greenblatt *et al.* [30].

Usually, the administration of these drugs to the patients is oral, however if a rapid action is required they can be administered by intravenous injection. 1,4-Benzodiazepines are absorbed from the gastrointestinal tract passing to the blood, where they are distributed throughout the organism. Clearly the rate of absorption will affect the response of an individual to drug therapy.

In most cases, the administered drug is the compound responsible for pharmacological action, however there are cases where the activity is due to some of the metabolites produced in the organism from the 1,4-benzodiazepine administered (e.g. Clorazepate, Prazepam and Flurazepam). In these cases, the 1,4-benzodiazepine administered is a precursor of the pharmacologically active compound. Sometimes the activity also is due to both the administered drug and its metabolite (e.g. Diazepam and Nordazepam).

The possible action of the active compound is a function of its concentration in the blood which in turn is dependant upon the amount of the dose administered and the mode of administration (oral, intravenous or intramuscular), the efficiency of absorption, its distribution to the tissues, and the velocity with which the active compound is metabolized.

After administration the blood levels of the active compound will increase due to the absorption of the drug or to its formation, if the active compound is a metabolite. At the same time, the distribution to the different tissues and metabolism of the active compound tend to decrease its blood level. Another phenomenon is the elimination of the active compound and its metabolites from the body through urine and to a minor extent the faeces. Thus, 70% of Diazepam is excreted in urine and 10% in faeces [12].

Table 3 shows some 1,4-benzodiazepines and their metabolites detected in blood and/or urine.

Table 3
1,4-Benzodiazepines and their metabolites detected in blood or urine and their binding to blood proteins

Drug	Metabolite	Detected in blood	Per cent protein binding	Detected in urine
Alprazolam		**17**, **30**, **31**	68.4 (**31**)	
	1-Hydroxymethylalprazolam	**17**		
Bromazepam				
	3-Hydroxybromazepam			**11**
	2-(2-Amino-5-bromobenzoyl)pyridine			**11**
	2-(2-Amino-5-bromo-3-hydroxy-benzoyl)pyridine			**11**
Camazepam	Temazepam			**11**
(Chlorazepate)		**17**		
	Nordazepam	**17**, **30**		**11**
	Oxazepam			**11**
Chlordiazepoxide		**17**, **30**, **32**, **33**, **34**	96.2 ± 0.24 (**32**) 94.7 ± 2.2 (**33**)	
	N-Desmethylchlordiazepoxide	**17**, **30**, **34**		**11**
	Demoxepam	**17**, **30**, **34**		**11**
	Nordazepam	minor (**30**), **34**		**11**
	Oxazepam	**34**		
Clobazam*		**16**, **17**, **30**, **31**	83.1 (**31**)	
	Desmethylclobazam	**16**, **17**, **30**		
Clonazepam		**13**, **17**		<0.5%† (**13**)
	7-Aminoclonazepam			**11**
	7-Acetamidoclonazepam			**11**
	3-Hydroxyclonazepam			**11**
	7-Amino-3-hydroxyclonazepam			**11**
Demoxepam				24–29%‡ (**22**), 41–45%§ (**22**)
	Opened lactam			2.8–5.5%‡ (**22**)
	Oxazepam conjugated			2.4–7.6%‡ (**22**)
	5-(4-Hydroxyphenyl)demoxepam			0–1.2%‡ (**22**)
	5-(4-Hydroxyphenyl)demoxepam conjugated			1.5–3%‡ (**22**)
	9-Hydroxydemoxepam conjugated			1.2–1.9%‡ (**22**)
	Desoxy-9-hydroxydemoxepam conjugated			1.1%‡ (**22**)
	N-Desoxydemoxepam (detected in faeces)			
Diazepam		**12**, **14**, **17**, major (**30**), **31**, **33**, **34**, **35**, **36**	99 (**30**), 98.4 (**31**), 95.5 ± 2.2 (**33**)	<0.05%‖ (**12**), **14**¶
	Nordazepam**	Major (**14**, **30**), **12**, **17**, **34**, **35**, **36**	98 (**30**)	<0.05%‖ (**12**), **14**¶, **11**
	Nordazepam conjugated			2.5–9.2%‖ (**12**)
	Oxazepam	Minor (**14**, **30**)		**14**¶
	Oxazepam glucuronide			Major (**12**, **30**)
	Temazepam	Minor (**14**, **30**)		**14**¶
	Temazepam glucuronide			**12**

Table 3
Continued

Drug	Metabolite	Detected in blood	Per cent protein binding	Detected in urine
Estazolam		**30**		
Flunitrazepam		**17**, **30**, **31**, **37**, **38**	77.5 (**31**)	**37**
	Norflunitrazepam	**17**, **30**, **38**		**11**
	7-Aminoflunitrazepam	**38**		**11**
	7-Amino-norflunitrazepam	**38**		
	3-Hydroxyflunitrazepam			**11**
(Flurazepam)		**17**, **39**		**39**
	***N*-Desalkylflurazepam**	**17**, major (**30**), **34**, **39**	97 (**30**)	**39**
	***N*-Hydroxyethylflurazepam**	**30**, **39**		**39**
	N-Hydroxyethylflurazepam (free and conjugated)	**17**		
	N-Hydroxyethylflurazepam glucuronide			40–55%†† (**39**)
	Flurazepam aldehyde	**30**		
Halazepam		**30**		
	Nordazepam	**30**		
Ketazolam		**17**		
	Diazepam	**17**		
	Nordazepam and/or *N*-desmethyl-ketazolam	**17**		
Lorazepam		**7**, **17**, **30**, **31**, **32**, **33**	93 (**7**), 89 (**30**), 90.3 (**31**), 93.6 ± 0.29 (**32**), 91.0 ± 2.2 (**33**)	
	Lorazepam glucuronide	**17**		**11**
Lormetazepam		17%‡‡ (**7**), **17**	88 (**7**)	<1.5%§§ (**7**)
	Lormetazepam glucuronide	83%‡‡ (**7**), **17**		76–99%§§(**7**)
	Lorazepam glucuronide	Traces (**7**)		4–16%§§ (**7**)
Medazepam		**17**		
	Diazepam	**17**		**11**
	Nordazepam	**17**, major (**30**)		**11**
	N-Desmethylmedazepam	**17**		**11**
	Oxazepam			**11**
	Dehydromedazepam			**11**
Midazolam		**30**, **31**	96.3 (**31**)	
Nitrazepam		**17**		
	2-Amino-5-nitrobenzophenone			**11**
	2-Amino-3-hydroxy-5-nitro-benzophenone			**11**
	7-Aminonitrazepam			**11**
	7-Acetamidonitrazepam			**11**

Nordazepam		**31**	96.5 (**31**)	**19**
	Oxazepam			**19**
Oxazepam		**17**, **30**, **31**, **33**, **34**	96 (**30**), 94.9 (**31**), 97.8 ± 2.3 (**33**)	
	Oxazepam glucuronide	**17**		**11**, 98.3% (**40**)
Pinazepam		**17**		
	Nordazepam	**17**		**19**
	Oxazepam			Major (**19**)
	3-Hydroxypinazepam			**19**
(Prazepam)		**17**		
	Nordazepam	**17**, **30**		**11**
	Oxazepam			**11**
Temazepam		**17**, **30**, **31**	97 (**30**), 96.4 (**31**)	<1% (**40**)
	Oxazepam			**11**, <1% (**40**)
	Temazepam glucuronide	**17**		86.6% (**40**)
	Oxazepam glucuronide			5.8% (**40**)
Triazolam		**8**, **30**, **31**, **41**	94 at 21°C and 89 at 37°C (**8**), 77.9 (**31**)	2.1%§§ (**8**)
	1-Hydroxymethyltriazolam glucuronide	Major (**8**), **30**		69%§§ (**8**)
	4-Hydroxytriazolam glucuronide	Major (**8**), **30**		11%§§ (**8**)
	1-Hydroxymethyltriazolam	Traces (**8**)		
	4-Hydroxytriazolam	Traces (**8**)		
	1-Hydroxymethyl-4-hydroxytriazolam glucuronide			2.3%§§ (**8**)
	5-Chloro-2-(3-methyl-4H-1,2,4-triazol-4-yl)-2′-chlorobenzophenone			0.8%§§ (**8**)
	5-Chloro-2-(3-hydroxymethyl-5-methyl-4H-1,2,4-triazol-4-yl)-2′-chlorobenzophenone			6%§§ (**8**)
	5-Chloro-2-(3,5-bis(hydroxymethyl)-4H-1,2,4-triazol-4-yl)-2′-chlorobenzophenone			3.5%§§ (**8**)

Compounds used in formulations are indicated below the column 'Drug', whilst their metabolites in man are shown below the label 'Metabolite'. Some 1,4-benzodiazepines can be found in both columns. Drugs and metabolites tested as pharmacologically active are in bold. Drugs that are precursors of other compound and do not show activity are in brackets. Relative importance of different metabolites of the same drug is indicated by the label 'Major', 'Minor' or its relative percentage. References are bold.

*Clobazam is a 1,5-benzodiazepine.

†Per cent of total dose, collected between 0 and 24 h.

‡Per cent of total dose, collected between 0 and 16 or 18 days.

§Percent of total metabolites, collected between 0 and 16 or 18 days.

‖Per cent of total dose, collected between 0 and 48 h.

¶After several weeks of treatment.

**Prefix nor- usually indicate N-demethylation.

††Per cent of total dose.

‡‡Per cent of total metabolites in blood.

§§Per cent of total metabolites, collected between 0 and 48 h.

Initially, the retention of the active compound predominates over its elimination. Thus, its blood concentration increases until it reaches a maximum level, C_{max}, at a time after administration, t_{max}. From this time, distribution to the tissues and elimination by the urine predominates and the blood level of the active compound decreases with a rate defined by the elimination half-life, $t_E^{1/2}$.

Usually, the treatment of patients is not in the form of single doses but a series of individual doses at various time intervals. This situation causes an accumulation of the active drug and its metabolites during the treatment. Thus, for a continuous treatment during 15 days with single doses of 10 mg of Diazepam per day C_{max} = 84–222 ng ml^{-1} and t_{max} = 7 days for Diazepam and C_{max} = 90–160 ng ml^{-1} and t_{max} = 7 days for Nordazepam [12]. Some metabolites difficult to detect in urine can be found during these long treatments. Thus, Nordazepam, Oxazepam and Temazepam can be detected in urine after several weeks of treatment with Diazepam [14].

Table 4 shows values of the mean absorption half-life, $t_A^{1/2}$, elimination half-life, $t_E^{1/2}$, and maximum blood level, C_{max} and t_{max}, for some 1,4-benzodiazepines and their metabolites.

Each individual metabolite will be detected in blood and/or urine depending on its particular characteristics and the metabolic reaction by means of which it has been originated. Metabolic reactions that different 1,4-benzodiazepines can undergo are numerous. Some of the more important being as follows:

(a) *Oxidation*

Many 1,4-benzodiazepines are biotransformed by oxidative reactions in the liver, primarily by demethylation or dealkylation of the nitrogen in position 1 and hydroxylation of the carbon in position 3. Some examples are:

Diazepam → Nordazepam (*N*-Desmethyldiazepam)
Lormetazepam → Lorazepam
Flurazepam → Desalkylflurazepam
Diazepam → Temazepam (3-Hydroxydiazepam)
Triazolam → 4-Hydroxy-1-hydroxymethyltriazolam

Table 4
Values of the mean absorption half-life, $t_A^{1/2}$, mean elimination half life, $t_E^{1/2}$, maximum blood level, C_{max}, and time at which this level is reached, t_{max}, for some 1,4-benzodiazepines and their metabolites. The dose for which C_{max} and t_{max} are given, is also indicated

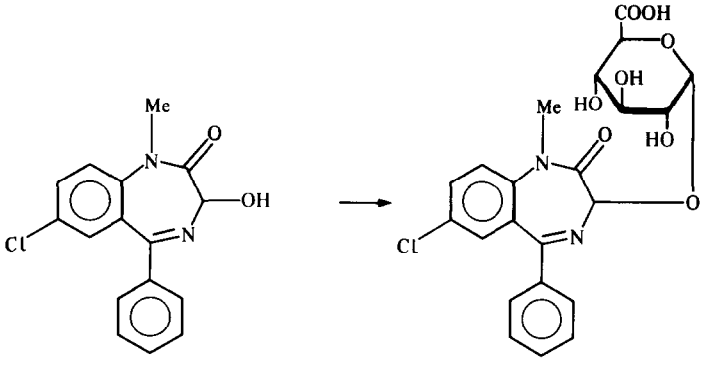
Drug	Metabolite	$t_A^{1/2}$ (h)	Dose*/C_{max}/t_{max} (mg/ng ml^{-1}/h)	$t_E^{1/2}$ (h)	Ref.
Alprazolam			Oral 1/25/1	11	30
			1/13.4/0.7–1.6	12–15	17
				15.9 ± 6.1	42
Bromazepam			12/131/1–4	11.9	17
Brotizolam				3.1–6.1	43
	1-Hydroxymethylbrotizolam			Rapid	43
(Chlorazepate)		Rapid	15/66/0.9	2	17
	Nordazepam		Oral 15/300/1.18	Slow	30
			15/230/1	64.8	17
Chlordiazepoxide		Medium			30
		0.83 ± 0.21		8.1 ± 0.93	32
			20/978/3.3	13.2	17
	N-Desmethylchlordiazepoxide		20/238/16	10–18	17
	Demoxepam		20/50/24	45	17
Clobazam†			20/465/1.7	24.5	17
				24	30
	Desmethylclobazem		20/88/45.6	36–46	17
Clonazepam			Oral 2/6.5–13/1–2	18.7–39.0	13
			2/10.4/2.6	26.4	17
Demoxepam				45 ± 14	22
Diazepam		Rapid	—/—/0.88	Slow	30
		Rapid	Oral 10/137–189/1–1.5	21–37	12
			iv 10/294–574/0.02–0.08		12
			Oral 20/490/0.5		35
			iv 20/1600/0.25		35
			im 20/290/1		35
	Nordazepam		Oral 10/26–37/30–48	50–99	12
			iv 10/19–39/30–48		12
			20/~100/72		35

Estazolam				Medium	30
Flunitrazepam			Oral 2/10–15/0.5		37
			2/8/2	20–35	17
				9–25	43
				Medium	30
	Norflunitrazepam		2/1.5/12		17
				31	43
	7-Aminoflunitrazepam			23	43
				13	38
(Flurazepam)			60/1–5/0.5–1	3.1	17
				Very rapid	43
	***N*-Desalkylflurazepam**		60/30–50/3	32–100	17
			Oral 15/10/24	82	30
				40–144	43
	***N*-Hydroxyethylflurazepam**		Oral 15/20/1		30
				Rapid	43
	N-Hydroxyethylflurazepam (free and conjugated)		60/150–250/1–4	3	17
	Flurazepam aldehyde		Oral 15/3/1		30
Halazepam				Slow	30
Ketazolam			30/4/2	1.5	17
	Diazepam		30/17/10	28	17
	Nordazepam and/or *N*-desmethyl-ketazolam		30/127/14	50	17
Loprazolam				4–8	43
Lorazepam		0.31 ± 0.06		8.9 ± 1.6	32
			2/23/2.57	15.87	17
	Lorazepam glucuronide		2/32.4/6.24	18.5	17
Lormetazepam			Oral 2/12.4 ± 5.4/2	13.2 ± 1.7	7
			iv 0.2/4.8 ± 3.5/0.03		7
			1/6.3/2.2	9.9	17
	Lormetazepam glucuronide		Oral 2/60/3		7
			iv 0.2/5/7		7
			1/34/6	12–13	17
Medazepam			30/141/1.5		17
	Diazepam		30/23/1–24		17
	Nordazepam		30/28–195/84		17
	N-Desmethylmedazepam		30/79/1.5		17
Midazolam				1–3	43
				2.5	30
	1-Hydroxymethylmedazepam			Rapid	43
Nitrazepam			5/40/2.3	29	17
				20–34	43
Oxazepam		Slow	Oral 30/500/4	8.5	30
			45/1089/2	3.9, 8	17
	Oxazepam glucuronide		45/999/3.4	5.6	17
			Oral 23.6/—/—	7.5 ± 2.1	40
Pinazepam			10/36.8/1.8	15.7	17
	Nordazepam		10/150/3		17
(Prazepam)		Slow			30
			30/6.6/0.5	1.3	17
	Nordazepam		Oral 20/50/7.8	Slow	30
			30/321/4.25	96	17
Temazepam		Slow	Oral 30/300/3	15	30
			20/668/0.75	8.3	17
			Oral 17.8/—/—	~3	40
	Temazepam glucuronide			1.9	17
			Oral 17.8/—/—	7.7 ± 1.7	40
	Oxazepam glucuronide		Oral 17.8/—/—	9.7 ± 2.4	40
Triazolam		0.13	—/0.72/—	4.5	41
		0.05	Oral 0.88/8.8/1.3	2.3	8
			Oral 0.5/5/1	3.1	30
	1-Hydroxymethyltriazolam glucuronide		Oral 0.88/6.1/1.3	3.9	8
	4-Hydroxytriazolam glucuronide		Oral 0.88/6.1/2.5	3.8	8
	1-Hydroxymethyltriazolam		—/1.2/—		8
	4-Hydroxytriazolam		—/0.9/—		8

*Single dose of administered drug: oral, iv (intravenous) or im (intramuscular).
†Clobazam is a 1,5-benzodiazepine.

(b) *Conjugation*

The 3-hydroxy substitution of some 1,4-benzodiazepines such as Oxazepam, Temazepam or Lorazepam allows direct conjugation to glucuronic acid, yielding pharmacologically inactive, water-soluble glucuronide conjugates that are excreted in urine:



Temazepam Glucuronide conjugate

Usually, an enzymatic hydrolysis is required in order to liberate 1,4-benzodiazepines from their conjugates.

(c) *Nitroreduction*

1,4-Benzodiazepines having 7-nitro substituents, such as Nitrazepam, Flunitrazepam and Clonazepam, are biotransformed by reduction of the nitro group to form biologically inactive 7-amino and 7-acetamido derivatives.

Table 3 shows the different metabolites tested for some 1,4-benzodiazepines.

Another factor to consider is that 1,4-benzodiazepines are extensively bound to plasma proteins. This fact affects the blood levels of the free drug. Due to this effect authors tend to refer to free and bound fractions of the total drug amount in blood. Protein binding of 1,4-benzodiazepines varies widely, ranging from 99% for Diazepam to less than 70% for Alprazolam, but is apparently not affected by drug concentration over the plasma levels encountered during usual therapeutic dosage [31, 33]. However, plasma binding of 1,4-benzodiazepines can be affected by the presence in the blood of other substances such as heparin [44]. Table 3 shows the extent of protein binding for different 1,4-benzodiazepines. A deproteination process is required to liberate the bound fraction of the drug from proteins.

Sample pretreatment

1,4-Benzodiazepines are usually present at trace levels ($\mu g\ ml^{-1}$ or $ng\ ml^{-1}$) in a complex biological matrix and the potentially interfering compounds need to be removed before analysis. Sample pretreatment should be capable of concentrating the sample and reducing the amount of interfering substances. Two reviews on sample pretreatment in the trace determination of drugs in biological fluids have been published [45, 46].

Collection and storage

Blood, like serum or plasma, and urine are the biological samples usually analysed. Other biological samples such as saliva [47], liver tissues [48] or erythrocytes [14] also have been analysed. Serum is the supernatant liquid collected by centrifugation after coagulation (about 30 min at room temperature) of a blood sample. Plasma is the supernatant liquid obtained by centrifugation of a blood sample collected in a tube containing an anticoagulant (e.g. heparin, EDTA, citrate or oxalate).

Plasma or serum samples can be kept for 6 h at room temperature, or for 1–2 days at 4°C. For longer storage periods samples should be frozen at −20°C. The way in which samples are collected and stored can affect the final results of the analysis, as has been demonstrated by Hoskin *et al.* in the case of morphine levels in blood [49].

Urine samples are taken as a single or as 8, 12, 24, 48, . . . h specimens. They must be stored by freezing at −20°C or by the addition of a preservative agent such as toluene, boric acid or concentrated hydrochloric acid [45].

Table 5 shows the different collection and storage conditions used by some authors.

Table 5
Some collection, storage, protein removal and enzymatic digestion conditions proposed for 1,4-benzodiazepines

Sample (ml)	Collection anticoagulant or preservative	Storage temperature (°C)	Protein precipitation agent/per cent drug released	Enzymatic hydrolysis enzyme/temperature/time/medium (°C) (h)	Ref.
U (200–250)				β-Glu-Sulph	22
P, U	Heparine	−18	Acetone:methanol (3:1)	β-Glu-Asulph/37/20/Ac O.1 M pH = 4.7	7
P, U	Heparine	−5			8
P (1)	Heparine		Dialysis		33
P	Heparine		Dialysis		31
P (0.1)	Citrate		Na octyl sulphate 0.2%		50
S, U (0.5–1)				β-Glu/37/5	51
U (10)				β-Glu/37/24/Ac 0.5 M pH = 5.0	52
U (0.05–0.5)*				β-D-Glu-Glu/37/24/Ac 0.2 M pH = 4.5	53
S (1)		−20			42
U				β-Glu/37/5/pH = 5.0 with HCl 2 M	47
Saliva	Centrifuge, decant				47
P (2)	Heparine	−20			54
Liver tissue (1–10 g)				Subtilisin/50–60/1/pH = 10.5 tris Base 1 M	48
S (0.2)			CH_3CN:HAc (4:1)/90–95		55
P (1), U (1), Erythrocytes (3)	EDTA	<0			14
U (0.2)†				Glusulase/37/2/NaAc 0.16 M pH = 5.3	56
B (1)			100°C and cool at r.t.		57
B (0.2–1)	EDTA	4			17
S (0.5)‡		−20			58
U				β-Glu-Asulph/55	59
S (1)		−20			16
U (1)				β-Glu-Sulph/37/12–18/Ph 1 M pH = 5.3	60
P or U (0.2)				β-Glu/—/24/0.067 M KH_2PO_4	40

P (plasma). S (serum). B (blood). U (urine). β-Glu-Sulph (β-Glucuronidase-Sulphatase or Glusulase). β-Glu-Asulph (β-Glucuronidase-Arylsulphatase). β-Glu (β-Glucurunidase). β-Glu-Glu (β-Glucuronide Glucuronosohydrolase). Ac (acetate buffer). Ph (Phosphate buffer).

*Mouse urine.

†Dog urine.

‡Calf serum.

Direct injection

Frozen samples of plasma, serum or urine should be brought to room temperature and mixed to ensure homogeneity before analysis. These samples can be directly injected into the HPLC column for analysis. For example, some drugs can be determined in urine by HPLC with direct on-column injection [61]. Some 1,4-benzodiazepines have been determined in serum by direct on-column injection after protein precipitation [55].

However, direct injection of complex samples leads to the contamination of columns impairing their performance. Contamination often persists even when a precolumn is used to protect the analytical column. To avoid these problems, sample clean-up is required. The most common procedures are enzymatic digestion, protein precipitation and solvent and solid-phase extractions.

1,4-Benzodiazepines are found in biological fluids as free molecules, conjugated as glucuronides or sulphates or bound to the proteins. Free drug, free and conjugated or total levels are measured depending on the pretreatment followed.

Enzymatic hydrolysis

Chemical hydrolysis of conjugates with hydrochloric acid or sodium hydroxide is not recommended for 1,4-benzodiazepines because they can be hydrolysed to the corresponding benzophenones in strongly acidic or basic media. Enzymatic digestion generally causes hydrolysis without degradation of the parent molecule to the corresponding benzophenone. An appropriate enzyme is mixed with the sample buffered at pH 5 and the mixture digested at 37°C for 24 h. However, Tjaden *et al.* [47] note that a hydrolysis period of longer than 5 h results in lower recoveries, owing to the low stability of the 1,4-benzodiazepines. β-Glucuronidase has been used to release 1,4-benzodiazepines from their conjugates with the glucuronic acid. Glusulase (β-Glucuronidase + Sulphatase) has been used to release 1,4-benzodiazepines from any type of conjugate. Table 5 gives an indication of the enzymatic hydrolysis conditions used by the various workers.

Protein removal

Protein removal from blood samples can be performed by various methods, some of which were checked by Blanchard [62]. Ultramicrofiltration and equilibrium dialysis [31, 33] of the sample remove proteins but the ultramicrofiltrate or the dialysate contains only the free fraction of the drug. Recently, an on-line dialysis process for protein removal in order to enable the automated HPLC analysis of drugs in body fluids has been described [63].

Usually, the method used for precipitation of plasma or serum proteins consists of mixing one volume of plasma or serum with three volumes of acid (6% m/v $HClO_4$, 10% m/v trichloroacetic acid) or organic solvent (ethanol, methanol, acetonitrile, acetone) followed by vortex-mixing and centrifugation [7, 45, 55]. This method removes 99% of the proteins [62] and releases the 1,4-benzodiazepines from protein-binding sites. Recoveries of the bound portion of drug are dependent upon the nature of the 1,4-benzodiazepine and the precipitation agent.

Another way to release 1,4-benzodiazepines from proteins without precipitation is the addition of fatty acids that compete with 1,4-benzodiazepines for binding sites of proteins [44] or the addition of alkyl sulphates such as sodium octylsulphate that disrupt the structure of proteins [50].

Table 5 shows the conditions used by some authors for protein removal in the analysis of 1,4-benzodiazepines.

Liquid–liquid extraction

Liquid–liquid extraction is the most widely used method for the pretreatment of biological samples. Usually a single extraction step involving 1 ml of sample and 5 ml of organic solvent may give over 80% recovery. However, some 1,4-benzodiazepines need a double extraction due to their low lipid solubility. Some workers recommend the use of a back-extraction with aqueous acid solutions and basifying followed by extraction with organic solvent. Complications can arise due to hydrolysis of the 1,4-benzodiazepines.

Solvent polarity and pH of the aqueous phases are the major factors to be considered. pH should be adjusted to a value at which the drug is in the neutral form but is not hydrolysed. 1,4-Benzodiazepines are usually extracted under weakly alkaline conditions, such as borate, phosphate or ammonium/ammonia buffer at pH about 9. Some workers have extracted 1,4-benzodiazepines at neutral pH or directly at physiological pH. The organic solvents usually chosen are diethyl ether,

chloroform, ethyl acetate, butyl acetate and others such as toluene, benzene, heptane or hexane to which a small amount of a more polar solvent such as methylene chloride, isoamyl alcohol or isopropanol is added.

Table 6 shows the conditions of liquid–liquid extraction procedures used for 1,4-benzodiazepines.

Usually a solvent evaporation step is required after extraction. The possible adsorption of the drug onto the glassware can be prevented by silanization of glassware or by the inclusion of 1–2% of alcohol (ethanol or 3-methylbutan-1-ol) in a non-polar extractant such as hexane or heptane [45].

Solid phase extraction

In solid phase extraction (SPE) the sample is poured directly onto a column packed with solid adsorbent (alumina, silica, chemically bonded silica, Florisil or non-ionic or ionic-exchange resins). Drugs are retained on the adsorbent surface. Undesirable compounds which are also adsorbed may be removed by washing with an appropriate solvent or buffer. Drugs and related compounds are then eluted by passing an appropriate elution solvent through the column.

Liquid–solid extraction can be performed with home-made columns, for example a Pasteur pipette packed with adsorbent. However, disposable columns of various sizes and with a wide range of adsorbents are commercially available. This method of sample clean-up is simpler, quicker and less laborious than liquid–liquid extraction. Some advantages of SPE are the minimal handling time, the high recovery even at low concentrations, the clean samples extracts and the little or no need for concentration of the extract. The efficiency and reproducibility are as good as those of liquid–liquid extraction.

Celite, activated Charcoal and Amberlite XAD-2 ion-exchange resin were used as adsorbent materials in the first studies on SPE for 1,4-benzodiazepines [78]. More recently, C-18 bonded-phase extraction columns have been used for the sample preparation of Diazepam, Chlordiazepoxide, and their metabolites [79, 80], Triazolam and metabolites [52] and for general 1,4-benzodiazepines [51]. Silica columns have been used for the sample pretreatment of Triazolam and metabolites in urine [52].

Three adsorbents (activated charcoal, Amberlite XAD-2 ion-exchange resin and Celite) have been compared for the extraction of Nitrazepam, Diazepam and Nordazepam [78]. Balkon *et al.* [81] have used Amberlite XAD-2 for the isolation of Diazepam from biological fluids. Porapak-T has been used by Lloyd and Parry [50] in connection with the determination of 1,4-benzodiazepines in plasma. Douse [17] has used Amberlite XAD-7 for trace analysis of 1,4-benzodiazepines in blood. Extrelut-20 has been used for the analysis of 1,4-benzodiazepines in urine [59].

Table 7 shows the solid-phase extraction procedures used by some authors.

Internal standard

It is usual to add a fixed amount of an internal standard to each sample at the earliest possible stage to permit correction for losses during sample treatment and to minimize errors due to variation in column separation or detector response. Most internal standards for 1,4-benzodiazepines are compounds of the same family. Thus, Etizolam has been used as internal standard for Triazolam and its metabolites [52], Alprazolam for 1-hydroxymethyltriazolam [53], Triazolam for Alprazolam [42] or Flurazepam for Midazolam and its metabolites [72]. However, in toxicological cases, a more suitable internal standard would be any 1,4-benzodiazepine that is not frequently encountered in such cases, such as Prazepam, recommended by many authors [16, 54, 66, 83]. Other compounds that are not 1,4-benzodiazepines, have been also used as internal standards such as chlorpromazine [68] and 6-nitroquinoline [50].

HPLC Analysis

Due to the complexity of biological samples, a chromatographic separation step is required for the analysis of drugs in such samples [46]. Chromatography can be avoided by the use of enzyme multiplied immunoassay techniques [84, 85], but they are not specific for each drug.

Thin-layer chromatography (TLC) is a valuable technique as an initial screening method to narrow the possible identities of unknown drugs in biological samples. However, it is relatively nonspecific, time consuming and provides only semiquantitative data. Gas chromatographic (GC) methods offer excellent sensitivity. However, they require somewhat lengthy clean-up procedures and, in

Table 6
Some liquid–liquid extraction conditions used for the separation of 1,4-benzodiazepines from biological samples

Sample (ml)	Buffer and pH	Solvent and relation/time/temperature/number of extns	Recovery (%)	Ref.
S (1)	Physiological	Benzene (1:3)	98 ± 11	35
U (200–250)	pH = 7 or pH = 2	Ethylacetate (1:1)/×2		22
P, U		Diethylether (4:1)/30 min/r.t.	83.4 ± 3.0 93.3 ± 2.1	7
P, U (1)		Chloroform (1:5)/30 seg		8
P* (1)	K phosphate 1 M pH = 9.0	Diethyl ether:methylene chloride 7:3 (3.1:8)/15 min	89–95	64
U† (0.05–0.5)	+ *x* ml acetate 0.2 M (pH = 4.5) + 2 ml NaOH 4 M	Methylene chloride:toluene 1:1 (*x* + 2:5)/15 min/×2	94.5	53
S (1)	+ 4 ml NaOH 4 M	Toluene (1:2)/15 min		42
S, P (2)	Physiological	Toluene:isoamyl alcohol 99:1 (1.3:5)/10 min		15
S, U, saliva (1)	+ 1 ml NH_4Cl/NH_3 sat. pH = 9.5 + 1 ml KCl sat.	10 ml diethylether/1 min/×2/Backextn/Reextn		47
P (1), U (3)	+ 1 ml borate sat. pH = 9.0	Chloroform (2:7)/10 min	81–91	65
P (2)	+ 2 ml borate pH = 9.0	Diethylether (2:5)/10 min	94–96	54
B (2)	+ 2 ml phosphate 1 M pH = 7.0	Diethylether (3:10)/5 min/Backextn/Reextn	70–80	66
P, U‡ (1)		*n*-Hexane (1:2)/×2	75 ± 3	67
B§ (0.05)	+125 µl 0.1 M NaOH pH = 9.0	Heptane: isoamyl alcohol 98.5:1.5 (7:120)/2 min	68–69	68
Liver tissue (1–10 g)	pH = 10.5	Diethylether (3:40)/×2	61–89	48
B (5–10)	Borate pH = 9.5	+40 ml diethylether		69
B (1)	+1 ml KCl sat.	Benzene (2:11)/30 min/Backextn/Reextn	92	70
P, U (1)	+2 ml NH_4Cl sat./NH_3 pH = 9.5	Toluene (2% isoamyl alc):heptane 4:1	100	14

Erythrocytes (3)		(3:5)/10 min		
B‡ (1)	+2 ml Na_3PO_4 sat. pH = 12.6	Benzene:methylene chloride 9:1 (3:8)/15 min	86 ± 5	56
U (0.2)	+2 ml phosphate 1 M pH = 11.0	Benzene:methylene chloride 9:1 (1.1:4)		56
B (2), U (5)	+5 ml borate pH = 9.0	+10 ml diethylether/10 min		71
B (1)	+ 2 ml borate 1 M pH = 9.0	Benzene:methylene chloride 9:1 (1:2)/5 min		57
B (0.2–1)	pH = 9.2	Toluene (1:5)/30 seg/×2		17
S (1–2)	+50 µl NaOH 1 M	+8 ml diethylether/10 min	93–100	72
U (2)	+3 ml NaOH 0.5 M	Diethylether (5:12)/5 min		73
S (0.2)	Borate sat. pH = 9.5	Toluene:heptane:isoamyl alcohol 76:20:4 (6:5)/1 min	99–107	74
S‖ (0.5)	Physiological	Methylene chloride:Cyclohexane 3:2 (1:5)	80–100	58
S (1)	+1 ml borate pH = 9.0	*n*-Hexane:methylene chloride:isopropanol 23:23:1 (1:2)	81–93	75
S, B (0.1)		Butyl-acetate	75–90	76
S (1)	+0.5 ml sat. Na_3PO_4	+5 ml methylene chloride/2 min	98.4–100	16
P (0.5–4)	+2 ml pH = 10 buffer	+ 10 ml diethylether (1% isoamyl alcohol)/15 min/ Backextn and Reextn	50–70	38
P or S (0.5)	+1 ml 1 M conc. NH_4OH pH = 11.0 (with conc. HAc)	$CHCl_3$:isopropanol 9:1 (3:10)/2 min	>95	34
P (0.5–1) or U (1)	+0.2–1 ml 1 M borate (pH = 9.0)	+5 ml benzene:methylene chloride (9:1)/10 min	60–93	60
P or U (0.1–0.2)	+1.2 ml 0.067 M KH_2PO_4	+2 ml diethyl chloride/1 min	89–96	40
P or S (0.25)	+0.5 ml sat. Na_3PO_4	+5 ml chloroform/20 min	89–106	36
U (10)	+1 M Ac (pH = 7)	+10 ml ethyl acetate/ / /×3	80 ± 4	77

The volume ratio between sample and organic solvent is indicated in brackets (aqueous phase:organic phase).
*Dog plasma.
†Mouse urine.
‡Dog urine.
§Mice blood.
‖Calf serum.

Table 7
Some solid phase extraction conditions for 1,4-benzodiazepines

Buffer and pH	Adsorbent	Washing solvent	Eluent solvent	Recovery (%)	Ref.
0.1 M Na_2HPO_4 Na octylsulphate 0.2%	Porapak-T	$CH_3CN:H_2O$ (1:8)	$CH_3CN:H_2O$ (7:4)	83.9–95.3	50
After enzymatic hydrolysis	RP-18 Baker		0.5 ml MeOH		51
Alkalinized with NH_3*	Sep-Pak C_{18}	5 ml H_2O, 5 ml H_2O:MeOH (8:2), 2 ml H_2O	7 ml CH_2Cl_2:MeOH (9:1)		52
Previous eluent dried + 5 ml CH_2Cl_2:MeOH (99:1)	Sep-Pak Silica	20 ml CH_2Cl_2, 25 ml CH_2Cl_2:MeOH (99:1)	20 ml CH_2Cl_2:MeOH (9:1)	~98	52
Dried + 20 µl Et_2O + 1 ml pentane†	Amberlite XAD-7 (1 mg)	1 ml Pentane × 3	100 µl Ethyl acetate × 3	61–95	17
	Extrelut-20		$CHCl_3$:MeOH (9:1)		59
S (1 ml)	Sep-Pak C_{18}	2 ml MeOH:H_2O (1:1)	2 ml MeOH	99.7 ± 1.3	25
S (1 ml) + 1 ml Ph pH = 7.0	Sep-Pak C_{18}	2 ml H_2O, 2 ml MeOH:H_2O (1:1)	2 ml $CHCl_3$	99	82
0.1 M Borate (pH = 9.5)	Bond-Elut C_{18}	H_2O × 2, 50 µl MeOH	200 µl MeOH, 100 µl MeOH	>88	80
0.1 M Na_2CO_3	Bond-Elut C_{18}	H_2O × 2, 50 µl MeOH	200 µl MeOH, 100 µl MeOH	91–97	79

*After enzymatic hydrolysis.
†After liquid–liquid extraction.

most cases, derivatization to more volatile compounds. The high temperatures required to elute 1,4-benzodiazepines, can lead to on-column decomposition, for instance Ketazolam [86], Oxazepam and Chlordiazepoxide [87].

HPLC offers an attractive analytical alternative for the routine determination of 1,4-benzodiazepines in biological samples. The extraction procedures are relatively simple. Formation of derivatives is not necessary if the detection system is UV spectrophotometry or amperometry, and it is possible to operate at ambient temperature. Moreover, if the detection system is not destructive, the eluted drugs can be easily recovered for further examination.

HPLC mechanisms (columns and mobile phases)

In the 1970s 1,4-benzodiazepines were separated on columns containing silica [67, 70, 86, 88, 89], anion-exchanger [77], cation-exchanger [90], Durapak OPN [86, 91] and Carbowax 400 coated supports [92] and detected by UV measurements detectors. Silica columns also have been used in the 1980s [42, 93, 94]. Factors affecting chromatographic separation of basic drugs, such as 1,4-benzodiazepines, on silica columns have been studied [95–97]. Recently, non-modified silica and alumina have been examined as weak cation-exchangers for basic drugs [98].

However, the most popular HPLC columns used for the analysis of 1,4-benzodiazepines in biological samples are reversed-phase columns. The packing materials used are silica chemically bonded with octadecyl, octyl, methyl, propyl, cyano, hexyl or phenyl groups. The mobile phases usually consist of a mixture of acetonitrile or methanol and water or aqueous acetate or phosphate buffer at weakly acidic pH. Chromatography normally is performed at room temperature under isocratic conditions, however in addition elevated temperatures (40–50°C) and under gradient elution conditions can be used.

The major problem in reversed-phase HPLC analysis of 1,4-benzodiazepines is peak tailing. To prevent this problem some authors add an organic modifier and/or a silanol deactivator to the mobile phase such as isopropanol [99], 1-propanol [50], THF [64], triethylamine (TEA) [58] or butylamine [73]. Other workers use ion-pair chromatography where penthane sulphonate [74] or methane sulphonate [100] is added to the mobile phase. Underberg-Chitoe *et al.* [98] propose the use of non-modified silica and alumina stationary phases in combination with water–methanol mixtures, because under these conditions silica and alumina have a weak cation-exchange behaviour.

Table 8 shows the chromatographic conditions used by some authors for the separation of 1,4-benzodiazepines.

Detection systems

The three most popular detectors for HPLC are UV, fluorimetric and electrochemical. The first detector system used for 1,4-benzodiazepines was UV spectrophotometry and still it is the most popular due to the high absorption of 1,4-benzodiazepines in the range of 200–240 nm. In some cases, 1,4-benzodiazepines are hydrolysed to their corresponding benzophenones before UV detection [11, 19, 69, 102]. Hydrolysis is usually performed in strong aqueous acid media (e.g. HCl or H_2SO_4 1–6 mol dm^{-3}) at high temperature (60–120°C) over various periods of time (0.5–12 h) [11]. The disadvantage of analysis of the 1,4-benzodiazepines through their benzophenones is that hydrolysis of different 1,4-benzodiazepines can produce the same benzophenone, with the corresponding lack of selectivity [19]. Recently, UV diode-array detectors have become commercially available. The sensitivity of this type of HPLC detector is still relatively low, but they can be useful for the identification of peaks and in order to establish their purity [106].

Fluorimetric detection has been less used. However, it can reduce the need of excessive clean-up due to its greater selectivity [46]. The native fluorescence of 1,4-benzodiazepines is very low [24, 38, 107, 108] and an adequate fluorescence intensity is only reached in strongly acidic media [24, 26, 82, 109]. The nature and concentration of the acid and the addition of an alcohol such as methanol or ethanol affect the fluorescence intensity [24, 82]. However, an excessive concentration of acid can lead to hydrolysis of the 1,4-benzodiazepine [24]. Another way to get a fluorescence enhancement is the use of low temperatures (77 K) [109, 110] or the use of a laser as excitation source [111].

Most of the assays based on fluorimetric detection of 1,4-benzodiazepines employ some form of derivatization reaction, such as transformation to the corresponding quinazolines

Table 8
Chromatographic conditions used for the separation of 1,4-benzodiazepines

Column	Mobile phase*	Flow (ml min^{-1})	T (°C)	Sample solvent	Injection volume (µl)	Chromatgraphic mode — detection†	Ref.
ODS-Hypersil 3 µm 150 × 4.5 mm	MeOH:1-propanol:Ph 0.02 M pH = 6 (100:7.5:80)	1	40	$CH_3CN:H_2O$ (7:4)	10	RP–EC	50
ODS-Hypersil 5 µm 200 × 5 mm	MeOH:H_2O:NaPh 0.1 M (55:25:20) pH = 7.25	1.5		H_2O:MeOH	20	RP–UV	93, 94
ODS-Hypersil 5 µm 200 × 5 mm	MeOH:H_2O:NaPh 0.1 M (70:10:20) pH = 7.67	1.5		H_2O:MeOH	20	RP–UV	93, 94
Radial Pak C_{18} 5 µm 100 × 5 mm	MeOH:NaCl 0.2 M (65:35)	1.2		Mobile phase	200	RP–EC	73
Radial Pak C_{18} 10 µm 100 × 8 mm	MeOH:Ph 10^{-2} M pH = 8.0 (65:35)	1	r.t.	Mobile phase	20	RP–UV	52
IBM C_{18} 5 µm 250 × 4.5 mm	MeOH:CH_3CN:Ph 10^{-2} M pH = 7.4:THF (29:28:41:2)	1.3		MeOH	10	RP–UV	64
Bio-Sil ODS-10 250 × 4 mm	CH_3CN:KPh 0.05 M pH = 4.5 (3:7)	2.5	45	Mobile phase	100	RP–UV	15
Spherisorb ODS 3 µm 250 × 2.6 mm	MeOH:H_2O (60:40)					RP–EC	101
Spherisorb-5-ODS 150 × 4.6 mm	MeOH:NaPh 0.025 M (60:40) pH = 7.8	1.0		EtOH		RP–UV	48
Spherisorb ODS 5 µm 250 × 4.6 mm	$CH_3CN:H_2O$ (53:47)	1.8		CH_3CN	10	RP–UV	16
Spherisorb ODS 10 µm 250 × 4.6 mm	MeOH:NaPh 0.025 M pH = 7.5 (56:44)	1.6		Mobile phase	10	RP–UV	65
Partisil-10 ODS 250 × 4.5 mm	CH_3CN:NaAc 0.01 M pH = 4.6 (35:65)	2.0	r.t.	EtOH	10–15	RP–UV	66
C_{18} Lichrosorb Si-100 10 µm 250 × 4 mm	MeOH:H_2O (6:4 to 10:0)‡	0.75				RP–UV	69
Alltech RP-C18 10 µm 250 × 4.6 mm	MeOH:H_2O (9:11) + 7 ml l^{-1} Butylamine pH = 3.1§	2			200	RP–EC	73
Supelco C-18 5 µm 250 × 4.5 mm	MeOH:CH_3CN:KPh 0.01 M pH = 6.0‖ (35:15:50)	1.0		Mobile phase	20	RP–UV	74

Supelco C-18 5 μm 250 × 4.5 mm	MeOH:CH_3CN:KPh 0.01 M pH = 6.0‖ (35:20:45)	1.0		Mobile phase	5–10	RP–UV	74
Zorbax ODS (5–6 μm) 250 × 4.6 mm	A:0.06 M NH_4OH pH = 7.69 B:0.06 M HAc, C:CH_3CN Gradient	2.0	50	MeOH	10	RP–UV	34
Ultrasphere ODS 5 μm 150 × 4.6 mm	CH_3CN:0.007 M K_2HPO_4 pH = 3.7 (with H_3PO_4) (7:13)	2.0	55	MeOH	15–30	RP–UV	79
μ-Bondapak C_{18} 10 μm 300 × 3.9 mm	MeOH:CH_3CN:Ph 10^{-2} M pH = 7.4:THF (30:28:40:2)	1.3		MeOH	10	RP–UV	64
μ-Bondapak C_{18} 10 μm 300 × 3.9 mm	MeOH:isopropanol:Ac 0.0075 M pH = 3.5 (53:5:42)	0.9		Isopropanol	10	RP–UV EC	99
μ-Bondapak C_{18} 10 μm 300 × 4 mm	Buffer pH = 8:CH_3CN (1:3)	2.5	r.t.	Mobile phase Acetone (5:1)	10–100	RP–Fl	38
μ-Bondapak C_{18} 10 μm 300 × 3.95 mm	MeOH:H_2O (11:9)	2.0		Mobile phase	25	RP–UV	60
μ-Bondapak C_{18} 300 × 4 mm	MeOH:H_2O (65:35)	2		MeOH	10–20	RP–UV	54
μ-Bondapak C_{18} 300 × 4 mm	KPh 10^{-3} M pH = 8:MeOH (6:4 to 1:9) 0–3 min	2.0		MeOH	20	RP–UV	68
μ-Bondapak C_{18} 300 × 4 mm or Ultrasphere ODS 5 μm 150 × 4.6 mm	CH_3CN:NaPh pH = 3.2 Grad: 5:95, 0 min; 22:78, 24 min 45:55, 34 min; 5:95, 49 min	3.0				RP–UV	55
Lichrosorb RP-8 5 μm	H_2O:CH_3CN (7:3)	2.0	50	Mobile phase	250	RP–UV	53
Lichrosorb RP8 5 μm 100 × 4.6 mm	H_2O:MeOH:CH_3CN (10:9:1)	1.6		Mobile phase	100	RP–UV	40
Lichrosorb RP-8 7 μm	MeOH:H_2O (1:1)	3.02	r.t.		10	RP–UV	102
Lichrosorb 10 RP8 250 × 4.6	H_2O:MeOH:TEA (43:57:0.25) pH = 6.0 with HAc 1 M	2			20–75	RP–UV	58
Lichrosorb 10 RP8 250 × 4.6 mm	CH_3CN:KPh 0.6% (1:1) pH = 3	2	r.t.	Mobile phase at pH = 5.6	20	RP–UV	75
Li-Chrosorb RP8 (10 μm) 250 × 4.6 mm	MeOH:H_2O (3:2)	4.0	40		10	RP–UV	103
Supelcosil LC 8 DB 5 μm 50 × 4.6 mm	MeOH:HAc 1% (2:3)	1.5	r.t.	MeOH	5	RP–UV	104
μ-Bondapak C_8 5 μm 120 mm	CH_3CN:Ac 10^{-3} M pH = 4.0 (1:1)					RP–Fl	51
Technicon Fast-LC-C-8 (RP 5 μm) 150 × 4.6 mm	MeOH:0.002 M KH_2PH_4:CH_3CN (53:46:1)	1.3	35	Mobile phase	10	RP–UV	80

Table 8
Continued

Column	Mobile phase*	Flow (ml min^{-1})	T (°C)	Sample solvent	Injection volume (µl)	Chromatgraphic mode — detection†	Ref.
Spherisorb C_6 (5 µm) 150 × 4.6 mm	CH_3CN:NaAc 0.1 M (2:3)	1.0	20	H_2O:CH_3CN (95:5)	50	RP–UV	105
CPS-Hypersil 100 × 4.5 mm	MeOH:1-propanol:Ph 0.02 M pH = 6 (100:7.5:80)	1	40	CH_3CN:H_2O (7:4)	10	RP–EC	50
Metilsilica 7–8 µm 100 × 2.8	MeOH:Ph 0.05 M pH = 6.0 (1:1)	0.56		Mobile phase	10–60	RP–UV	47
Metilsilica 7–8 µm 100 × 2.8	MeOH:Ph 0.05 M pH = 7.0 (2:3)	1.82		Mobile phase	10–60	RP–UV	47
Spherisorb 5 CN 150 × 5 mm	MeOH:2-propanol (75:25) with 0.02% $HClO_4$	3		MeOH	20	RP–UV	72
µ-Bondapak CN	CH_3CN:KPh pH = 4.5 (7:13)	1.5	r.t.	Mobile phase	50	RP–UV	36
Lichrosorb Hibar 7 µm 250 × 4 mm	MeOH:H_2O (1:1)	3.02	r.t.	MeOH	10	RP–UV	19
Zorbax SIL 6 µm 250 × 4.6 mm	CH_3CN:H_2O (96:4)	1.5	r.t.	Acetone:CH_3CN (1:5)	250	NP–UV	42
Spherisorb S5W 5 µm 250 × 5 mm	$HClO_4$ 72% (0.1 ml):MeOH (1000 ml)	2.0		MeOH	20	NP–UV	93, 94
Spherisorb S5W 5 µm 250 × 5 mm	F_3CCOOH:H_2O:MeOH (1:2:997)	2.0		MeOH	20	NP–UV	93, 94
Spherisorb S-W 10 250 × 3 mm	*n*-Hexane + 5% EtOH or *n*-Hexane + 3% EtOH	2		*n*-Hexane	100	NP–UV	67
Partisil 10 250 × 4.6 mm	*n*-Heptane:isopropanol: MeOH (40:10:1)	1	r.t.	Mobile phase	40–80	NP–UV	70
Corasil II	THF:isopropylic ether (15:85)	1	26		1–4	NP–UV	86
Durapak-OPN/Poracil C	THF:isopropylic ether (10:90)	1.25	26		1–4	NP–UV	86
Zipax SAX 30 µm 500 × 2 mm	Ethyl acetate:hexane (3:7)	1.0		Ethyl acetate		IE–UV	77

*KPh = phosphate buffer with K^+ as counter-ion. NaPh = phosphate buffer with Na^+ as counter-ion. Ac = acetate buffer. When pH value is before relation of different solvents refers to the pH of the buffer and when is after is the final pH of the mobile phase.

†RP = reversed phase. NP = normal phase. IE = ion exchange.

‡According to the different 1,4-benzodiazepines.

§Adjusted with H_2SO_4.

‖With 2.6 g l^{-1} of pentane sulphonic acid sodium salt.

[25, 111] or acridanones [39, 51, 110, 112–114], or the preparation of derivatives by reaction with fluorescamine [38, 115, 116]. Photolytic rearrangement of chlordiazepoxide in alkaline medium gives a derivative containing 4,5-epoxide group which exhibits an intense fluorescence [107, 117]. This reaction is specific for 1,4-benzodiazepines with a *N*-oxide group. Oxazepam exhibits a very intense fluorescence in alcoholic phosphoric acid solutions after mild heating due to the formation of a dimer [118–120]. Other possible derivatization reactions are reported by Brooks and de Silva [121] and Gasparic and Zimak [11].

The presence of reducible groups in 1,4-benzodiazepines allows their electrochemical detection. All 1,4-benzodiazepines have the 4,5-azomethine group. Furthermore, most of them have other electroactive groups such as nitro, *N*-oxide or hydroxy groups [20, 21, 28, 121]. Reductions or oxidations are usually performed on carbon and mercury electrodes. Electrochemical detection has been used less in HPLC due to the possible contamination of electrodes and the need for a small cell volume. However, advances in electrode design have enabled the use of these detectors in the last years. Lund *et al.* [101] have designed a simple flow cell with interchangeable working electrodes made from glassy carbon, carbon paste and mercury. A detection limit of 3 ng was achieved for Nitrazepam and 300 ng for Diazepam and Chlordiazepoxide using the glassy carbon electrode.

The ideal working electrode material should have large anodic and cathodic potential ranges with low background currents and the properties of the electrode surface should not change with time. Glassy carbon and carbon paste electrodes are particularly well suited for detection in the anodic range.

Electrodes made from glassy carbon have a relatively large cathodic range, but need a cumbersome polishing procedure, while carbon paste electrodes exhibit a particularly low background in the anodic region and a fresh electrode surface can be obtained by simply removing the top layer of the carbon paste [101]. A comparison between UV and amperometric detection for the HPLC analysis of several types of drugs has been performed by Musch *et al.* [122].

The mercury pool electrode has a similar cathodic range and background as carbon electrodes [101]. Hanekamp *et al.* [123] have used a pulse-polarographic detector with a Dropping Mercury Electrode (DME) for the detection of four 1,4-benzodiazepines in a mixture. Hackman and Brooks [99] have determined Chlordiazepoxide and Desmethylchlordiazepoxide in plasma using reductive electrochemical detection at a DME operated in the differential pulse mode. The DME has the advantage of presenting a continuously renewable fresh surface during chromatographic analysis and so it is not subject to poisoning as are solid electrodes [99]. Recently, Lloyd and Parry [50] have reported the use of a Hanging Mercury Drop Electrode (HMDE) for the reductive electrochemical detection of 1,4-benzodiazepines in blood samples. They note that the installation of an upstream coulometric detector with both of the porous carbon working electrodes at 0 V lowers the background current at the mercury electrode to 2–6 nA.

Reductive detection in all cases require the removal of oxygen from the mobile phase and sample.

Oxidation of 1,4-benzodiazepines has been less studied. The oxidation occurs at the nitrogen atom of the 1,2-amide group and produces a *N*-oxide. In other cases oxidation also occurs through a nitrogen atom of an amino substituent, as in Flunitrazepam. Carbon electrodes are the most commonly used for oxidations due to their greater anodic range. Selavka *et al.* [73] have described an on-line post-column continuous photolytic derivatization system previous to the oxidative electrochemical detection at a glassy carbon electrode.

Table 9 shows the conditions used for the detection of 1,4-benzodiazepines by different authors.

Conclusion

This review has presented a general vision of the multiple HPLC methods available for the determination of 1,4-benzodiazepines in biological samples. Some important physicochemical and biopharmaceutical data have been collected to aid in the development of new HPLC methods.

Most of the HPLC methods reported have an acceptable sensitivity and should provide a useful alternative to GC methods. The clean-up procedure required depends upon the format of the analyte: free drug, its conjugated

Table 9
Detection systems and internal standards used for analysis of 1,4-benzodiazepines in biological samples

Detection system	Internal standard	Compound detected	Ref.
UV (202)	U-31485	Alprazolam and met.	15
UV (210)		Benzodiazepines and other drugs	55
UV (214)	Alprazolam	Triazolam and met.	53
UV (214)	Triazolam	Alprazolam	42
UV (215)	Flurazepam	Midazolam and 1-Hydroxymidazolam	72
UV (220)	Etizolam	Triazolam and met.	52
UV (228)	Prazepam or Heptabarbitone	Clobazam, Desmethylclobazam	16
UV (230)	Diazepam	Flunitrazepam and met.	67
UV (230)	Flunitrazepam	Diazepam, Oxazepam, Temazepam and Nordazepam	40
UV (232)		Diazepam and Nordazepam	70
UV (240)		Benzodiazepines	93, 94
UV (240)	Methylnitrazepam	Diazepam, Oxazepam, Temazepam and Nordazepam	80
UV (240)	Prazepam	Diazepam, Oxazepam and Nordazepam	66
UV (242)	Nitrazepam	Chlordiazepoxide and met., Diazepam and met.	79
UV (254)		Benzodiazepines	74
UV (254)		Benzodiazepines	75
UV (254)		Ketazolam, Diazepam, Oxazepam and Nitrazepam	86
UV (254)		Diazepam, Oxazepam, Temazepam and Nordazepam	60
UV (254)	Iodo analogue	Midazolam and met.	64
UV (254)		Benzodiazepines	47
UV (254)	Chlorpromazine	Chlordiazepoxide and met.	68
UV (254)		Benzodiazepines and Benzophenones	48

UV (254)		Benzodiazepines and Benzophenones	69
UV (254)	2-Amino-2,5-dichlorobenzophenone	Chlordiazepoxide and met., Diazepam and met., Prazepam, Desalkylflurazepam	34
UV (254)	Camazepam	Benzophenones	102
UV (254)	Camazepam	Benzophenones	19
UV (254)	Chlordiazepoxide	Diazepam, Oxazepam and Nordazepam	36
UV (254)	Diazepam	Chlordiazepoxide and met.	65
UV (254)	Medazepam	Chlordiazepoxide and met.	99
UV (254)	*N*-Methylnitrazepam	Diazepam, Chlordiazepoxide and met.	58
UV (254)	Prazepam	Diazepam and Nordazepam	54
UV (254)	Sulphanilamide	Temazepam	104
UV (260)		Nitrazepam and met.	77
UV (313)		Clonazepam	74
Fl (260, 430)		Acridanones	51
Fl (390, 470)	7-Aminomethylclonazepam fluorescamine derivative	7-Aminonorflunitrazepam and 7-Aminoflunitrazepam derivs.	38
EC (HMDE, −1.2 vs Ag/AgCl)	6-Nitroquinoline	Benzodiazepines	50
EC (−0.82 vs Ag/AgCl)	Medazepam	Chlordiazepoxide and met.	99
EC (Carbon, −0.93 vs Ag)		Nitrazepam, Diazepam and Chlordiazepoxide	101
EC (Hg pool)		Nitrazepam, Diazepam and Chlordiazepoxide	101
EC (Carbon, +0.75 and +1.1)		Benzodiazepines and other drugs	73

UV = Ultraviolet detection (λ is indicated in nm).
Fl = Fluorescence detection (λ_{exc} and λ_{em} are indicated in nm).
EC = Electrochemical detection (electrode and applied potential are indicated).
HMDE = Hanging mercury drop electrode.
met. = Metabolites.

form, or protein bound fraction of the 1,4-benzodiazepine of interest. Equilibrium dialysis or ultramicrofiltration should be used for the measurement of the free fraction. Enzymatic hydrolysis should be used for the measurement of the free and conjugated portions. Both enzymatic hydrolysis and protein precipitation, when a measurement of the total amount of the drug is required. After this previous treatment liquid–liquid or solid-phase extractions are necessary. SPE offers some advantages because it is simpler, quicker and less laborious than liquid–liquid extraction.

The HPLC columns most used are C18 reversed-phase. However, other packing materials, addition of organic modifiers or other chromatographic mechanisms could be considered for reducing peak tailing.

The most popular detector is UV, however there is increasing interest in electrochemical detection in recent years. New studies on this subject are still necessary.

Acknowledgements — The authors wish to thank Dr Luis Angel Fernández for his kind collaboration in the preparation of the manuscript. These studies were supported by the Dirección General de Investigación Científica y Técnica (DGICYT, project PB87-0733).

References

[1] J.M. Clifford and W.F. Smyth, *Analyst* **99**, 241–272 (1974).
[2] A.C. Mehta, *Talanta* **31**, 1–8 (1984).
[3] M.A. Peat, in *High Performance Liquid Chromatography in Forensic Chemistry* (I.S. Lurie and J.D. Wittwer, Eds), Ch. 6. Marcel Dekker, New York (1983).
[4] H. Schütz, *Benzodiazepines. A Handbook. Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature*. Springer-Verlag, Berlin (1982).
[5] H. Schütz, *Benzodiazepines II. A Handbook. Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature*. Springer-Verlag, Berlin (1989).
[6] P. Seiler and I. Zimmermann, *Arznein.-Forsch.* **33**, 1519–1522 (1983).
[7] M. Hümpel, V. Illi, W. Milius, H. Wendt and M. Kurowski, *Eur. J. Drug Metab. Pharmacokinet.* **4**, 237–243 (1979).
[8] F.S. Eberts, Y. Philopoulos, L.M. Reineke and R.W. Vliek, *Clin. Pharmacol. Ther.* **29**, 81–93 (1981).
[9] J.T. Carstensen, K.S.E. Su, P. Maddrell, J.B. Johnson and H.N. Newmark, *Bull. Parenteral Drug Assoc.* **25**, 193–202 (1971).
[10] M. Windholz and S. Budavari (Eds), *The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologicals*. Merck & Co., Rahway, NJ (1983).
[11] J. Gasparic and J. Zimak, *J. Pharm. Biomed. Anal.* **1**, 259–279 (1983).
[12] S.A. Kaplan, M.L. Jack, K. Alexander and R.E. Weinfeld, *J. Pharm. Sci.* **62**, 1789–1796 (1973).
[13] S.A. Kaplan, K. Alexander, M.L. Jack, C.V. Puglisi, J.A.F. de Silva, T.L. Lee and R.E. Weinfeld, *J. Pharm. Sci.* **63**, 527–532 (1974).
[14] I.A. Zingales, *J. Chromatogr.* **75**, 55–78 (1973).
[15] S.R. McCormick, J. Nielsen and P. Jatlow, *Clin. Chem.* **30**, 1652–1655 (1984).
[16] N. Ratnaraj, V. Goldberg and P.T. Lascelles, *Analyst* **109**, 813–815 (1984).
[17] J.M.F. Douse, *J. Chromatogr.* **301**, 137–154 (1984).
[18] E. Roets and J. Hoogmartens, *J. Chromatogr.* **194**, 262–269 (1980).
[19] C. Violon, L. Pessemier and A. Vercruysse, *J. Chromatogr.* **236**, 157–168 (1982).
[20] J. Barrett, W.F. Smyth and J.P. Hart, *J. Pharm. Pharmacol.* **26**, 9–17 (1974).
[21] J.M. Clifford and W.F. Smyth, *Fresenius Z. Anal. Chem.* **264**, 149–153 (1973).
[22] M.A. Schwartz and E. Postma, *J. Pharm. Sci.* **61**, 123–125 (1972).
[23] A. MacDonald, A.F. Michaelis and B.Z. Senkowski, in *Analytical Profiles of Drug Substances* (K. Florey, Ed.), Vol. 1, pp. 79–100. Academic Press, New York (1972).
[24] J.R. Procopio, P. Hernandez, M.T. Sevilla and L. Hernandez, *Anales de Química* **82**, 317–321 (1986).
[25] J. Rodriguez Procopio, P. Hernandez Hernandez and L. Hernandez Hernandez, *Analyst* **112**, 79–82 (1987).
[26] D.D. Maness and G.J. Yakatan, *J. Pharm. Sci.* **64**, 651–654 (1975).
[27] K. Florey (Ed.), *Analytical Profiles of Drug Substances*, Vols 1, 3, 4, 6, 9 and 16. Academic Press, New York (1972, 1974, 1975, 1977, 1980 and 1987).
[28] M.A. Brooks, J.J. Del Bruno, J.A.F. de Silva and M.R. Hackman, *Anal. Chim. Acta* **74**, 367–385 (1975).
[29] G. Klopman and R. Contreras, *Mol. Pharmacol.* **27**, 86–93 (1985).
[30] D.J. Greenblatt, M. Divoll, D.R. Abernethy, H.R. Ochs and R.I. Shader, *Drug Metab. Rev.* **14**, 251–292 (1983).
[31] L.J. Moschitto and D.J. Greenblatt, *J. Pharm. Pharmacol.* **35**, 179–180 (1983).
[32] J.W. Secor, K.V. Speeg, C.G. Meredith, R.F. Johnson, P. Snowdy and S. Schenker, *Br. J. Clin. Pharmacol.* **20**, 710–713 (1985).
[33] R.F. Johnson, S. Schenker, R.K. Roberts, P.V. Desmond and G.R. Wilkinson, *J. Pharm. Sci.* **68**, 1320–1322 (1979).
[34] G.L. Lensmeyer, C. Rajani and M.A. Evenson, *Clin. Chem.* **28**, 2274–2278 (1982).
[35] L. Hillestad, T. Hansen, H. Melsom and A. Drivenes, *Clin. Pharmacol. Ther.* **16**, 479–484 (1974).
[36] J.J. Mackichan, W.J. Jusko, P.E. Duffner and M.E. Cohen, *Clin. Chem.* **25**, 856–859 (1979).
[37] J.P. Cano, M. Soliva, D. Hartmann, W.H. Ziegler and R. Amrein, *Arzneim.-Forsch.* **27**, 2383–2388 (1977).
[38] Y.C. Sumirtapura, C. Aubert, Ph. Coassolo and J.P. Cano, *J. Chromatogr.* **232**, 111–118 (1982).
[39] J.A.F. de Silva, I. Bekersky and C.V. Puglisi, *J. Pharm. Sci.* **63**, 1837–1841 (1974).
[40] T.B. Vree, A.M. Baars, Y.A. Hekster, E. Van der Kleijn and W.J. O'Reilly, *J. Chromatogr.* **162**, 605–614 (1979).
[41] C.M. Metzler, H. Ko, M.E. Roger, W. Veldkamp and O.I. Linet, *Clin. Pharmacol. Ther.* **21**, 111–112 (1977).
[42] W.J. Adams, P.A. Bombardt and J.E. Brewer, *Anal. Chem.* **56**, 1590–1594 (1984).
[43] D.D. Breimer and R. Jochemsen, *Br. J. Clin. Pharmacol.* **16**, 277S–278S (1983).

[44] P.V. Desmond, R.K. Roberts, A.J.J. Wood, G.D. Dunn, G.R. Wilkinson and S. Schenker, *Br. J. Clin. Pharmacol.* **9**, 171–175 (1980).
[45] A.C. Mehta, *Talanta* **33**, 67–73 (1986).
[46] J.A.F. de Silva, *J. Chromatogr.* **273**, 19–42 (1983).
[47] U.R. Tjaden, M.T.H.A. Meeles, C.P. Thys and M. Van der Kaay, *J. Chromatogr.* **181**, 227–241 (1980).
[48] M.D. Osselton, M.D. Hammond and P.J. Twitchett, *J. Pharm. Pharmacol.* **29**, 460–462 (1977).
[49] P.J. Hoskin, O. Alsayed-Omar, G.W. Hanks, A. Johnston and P. Turner, *Ann. Clin. Biochem.* **26**, 182–184 (1989).
[50] J.B.F. Lloyd and D.A. Parry, *J. Chromatogr.* **449**, 281–297 (1988).
[51] N. de Giovanni and M. Chiarotti, *J. Chromatogr.* **428**, 321–329 (1988).
[52] T. Inoue and S.-I. Suzuki, *J. Chromatogr.* **422**, 197–204 (1987).
[53] W.J. Adams, P.A. Bombardt and R.A. Code, *J. Pharm. Sci.* **72**, 1185–1189 (1983).
[54] R.R. Brodie, L.F. Chasseaud and T. Taylor, *J. Chromatogr.* **150**, 361–366 (1978).
[55] P.M. Kabra, B.E. Stafford and L.J. Marton, *J. Anal. Toxicol.* **5**, 177–181 (1981).
[56] C.V. Puglisi, J.C. Meyer, L. D'Arconte, M.A. Brooks and J.A.F. de Silva, *J. Chromatogr.* **145**, 81–96 (1978).
[57] J.A.F. de Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfeld, *Anal. Chem.* **48**, 10–19 (1976).
[58] H.J.J. Willems, A. Van der Horst, P.N.F.C. de Goede and G.J. Haakmeester, *Pharm. Weekbl., Sci. Ed.* **7**, 150–157 (1985).
[59] H. Schütz, W.-R. Schneider and K. Schölermann, *Aerztl. Lab.* **34**, 130–136 (1988).
[60] S. Cotler, C.V. Puglisi and J.H. Gustafson, *J. Chromatogr.* **222**, 95–106 (1981).
[61] P. Lagerström, *J. Chromatogr.* **225**, 476–481 (1981).
[62] J. Blanchard, *J. Chromatogr.* **226**, 455–460 (1981).
[63] U.R. Tjaden, E.A. De Bruijn, J. Van der Greef and H. Lingeman, *LC–GC International* **1**, 32–42 (1988).
[64] C.V. Puglisi, J. Pao, F.J. Ferrara and J.A.F. de Silva, *J. Chromatogr.* **344**, 199–209 (1985).
[65] M.A. Peat, B.S. Finkle and M.E. Deyman, *J. Pharm. Sci.* **68**, 1467–1468 (1979).
[66] P.M. Kabra, G.L. Stevens and L.J. Marton, *J. Chromatogr.* **150**, 355–360 (1978).
[67] T.B. Vree, B. Lenselink and E. Van der Kleijn, *J. Chromatogr.* **143**, 530–534 (1977).
[68] H.B. Greizerstein and C. Wojtowicz, *Anal. Chem.* **49**, 2235–2236 (1977).
[69] K. Harzer and R. Barchet, *J. Chromatogr.* **132**, 83–90 (1977).
[70] A. Bugge, *J. Chromatogr.* **128**, 111–116 (1976).
[71] J.A.F. de Silva, C.V. Puglisi and N. Munno, *J. Pharm. Sci.* **63**, 520–527 (1974).
[72] A. Blackett, S. Dhillon, J.A. Cromarty, R. Horne and G. Richards, *J. Chromatogr.* **433**, 326–330 (1988).
[73] C.M. Selavka, I.S. Krull and I.S. Lurie, *J. Chromatogr. Sci.* **23**, 499–508 (1985).
[74] V.M. Haver, W.H. Porter, L.D. Dorie and J.R. Lea, *Ther. Drug Monit.* **8**, 352–357 (1986).
[75] P. Hochmuth, J.L. Robert, H. Schreck and R. Wennig, *Anal. Chem. Symp. Ser.* **20**, 143–149 (1984).
[76] L. Ulrich and F. Sager, *Beitr. Gerichtl. Med.* **46**, 135–141 (1988).
[77] B. Moore, G. Nickless, C. Hallett and A.G. Howard, *J. Chromatogr.* **137**, 215–217 (1977).
[78] A.W. Missen, *Clin. Chem.* **22**, 927–928 (1976).
[79] T.J. Good and J.S. Andrews, *J. Chromatogr. Sci.* **19**, 562–566 (1981).
[80] S.N. Rao, A.K. Dhar, H. Kutt and M. Okamoto, *J. Chromatogr.* **231**, 341–348 (1982).
[81] J. Balkon, B. Donnelly and D. Prendes, *J. Forensic Sci.* **27**, 23–31 (1982).
[82] F. Urrea Sanchez, J. Rodriguez Procopio and L. Hernandez Hernandez, *Anal. Chim. Acta* **199**, 227–232 (1987).
[83] C.B. McAllister, *J. Chromatogr.* **151**, 62–66 (1978).
[84] R.H. Drost, T.A. Plomp and R.A.A. Maes, *Clin. Toxicol.* **19**, 303–312 (1982).
[85] J.E. Wallace, S.C. Harris and E.L. Shimek, *J. Clin. Chem.* **26**, 1905–1907 (1980).
[86] D.J. Weber, *J. Pharm. Sci.* **61**, 1797–1800 (1972).
[87] W. Sadee and E. Van der Kleijn, *J. Pharm. Sci.* **60**, 135–137 (1971).
[88] C. Gonnet and J.L. Rocca, *J. Chromatogr.* **120**, 419–433 (1976).
[89] D.H. Rodgers, *J. Chromatogr. Sci.* **12**, 742–746 (1974).
[90] P.J. Twitchett, A.E.P. Gorvin and A.C. Moffat, *J. Chromatogr.* **120**, 359–368 (1976).
[91] C.G. Scott and P. Bommer, *J. Chromatogr. Sci.* **8**, 446–448 (1970).
[92] K. Macek and V. Rehak, *J. Chromatogr.* **105**, 182–185 (1975).
[93] R. Gill, B. Law and J.P. Gibbs, *J. Chromatogr.* **356**, 37–46 (1986).
[94] M. Japp, K. Garthwaite, A.V. Geeson and M.D. Osselton, *J. Chromatogr.* **439**, 317–339 (1988).
[95] R.J. Flanagan, G.C.A. Storey, R.K. Bhamra and I. Jane, *J. Chromatogr.* **247**, 15–37 (1982).
[96] R.J. Flanagan and I. Jane, *J. Chromatogr.* **323**, 173–189 (1985).
[97] I. Jane, A. McKinnon and R.J. Flanagan, *J. Chromatogr.* **323**, 191–225 (1985).
[98] U.K. Underberg-Chitoe, W.J.M. Underberg and H. Lingeman, *Anal. Sci.* **4**, 91–96 (1988).
[99] M.R. Hackman and M.A. Brooks, *J. Chromatogr.* **222**, 179–190 (1981).
[100] C.J. Sohr and A.T. Buechel, *J. Anal. Toxicol.* **6**, 286–289 (1982).
[101] W. Lund, M. Hannisdal and T. Greibrokk, *J. Chromatogr.* **173**, 249–261 (1979).
[102] C. Violon and A. Vercruysse, *J. Chromatogr.* **189**, 94–97 (1980).
[103] H. Haberstumpf, U. Mayer and K. Goßler, *Fresenius Z. Anal. Chem.* **307**, 400–403 (1981).
[104] A.A. Fatmi and E.A. Hickson, *J. Pharm. Sci.* **77**, 87–89 (1988).
[105] V. Rovei and M. Sanjuan, *Ther. Drug Monit.* **2**, 283–287 (1980).
[106] L. Huber and K. Zech, *J. Pharm. Biomed. Anal.* **6**, 1039–1043 (1988).
[107] N. Strojny, K. Bratin, M.A. Brooks and J.A.F. de Silva, *J. Chromatogr.* **143**, 363–374 (1977).
[108] J.A.F. de Silva, N. Munno and N. Strojny, *Anal. Chem.* **45**, 665–671 (1973).
[109] L.A. Gifford, J.N. Miller, J.W. Bridges and D.T. Burns, *Talanta* **24**, 273–275 (1977).
[110] J.A.F. de Silva, N. Strojny and K. Stika, *Anal. Chem.* **48**, 144–155 (1976).
[111] N. Strojny and J.A.F. de Silva, *Anal. Chem.* **52**, 1554–1559 (1980).
[112] J.A.F. de Silva and N. Strojny, *J. Pharm. Sci.* **60**, 1303–1314 (1971).
[113] J.C. Valentour, J.R. Monforte, B. Lorenzo and I. Sunshine, *Clin. Chem.* **21**, 1976–1979 (1975).
[114] P. Lafargue, J. Meunier and Y. Lemontey, *J. Chromatogr.* **62**, 423–429 (1971).
[115] J.T. Stewart and J.L. Williamson, *Anal. Chem.* **48**, 1182–1185 (1976).

[116] J.A.F. de Silva and N. Strojny, *Anal. Chem.* **47**, 714–718 (1975).
[117] M.A. Schwartz and E. Postma, *J. Pharm. Sci.* **55**, 1358–1362 (1966).
[118] J. Braun, G. Caille and G.A. Martin, *Can. J. Pharm. Sci.* **3**, 65–68 (1968).
[119] G. Caille, J. Braun and J.A. Mockle, *Can. J. Pharm. Sci.* **5**, 78–80 (1970).
[120] J. Gasparic, J. Zimak, P. Sedmera, Z. Breberova and J. Volke, *Coll. Czech. Chem. Commun.* **44**, 2243–2249 (1979).
[121] M.A. Brooks and J.A.F. de Silva, *Talanta* **22**, 849–860 (1975).
[122] G. Musch, M. De Smet and D.L. Massart, *J. Chromatogr.* **348**, 97–110 (1985).
[123] H.B. Hanekamp, W.H. Voogt, R.W. Frei and P. Bos, *Anal. Chem.* **53**, 1362–1365 (1981).

[*Received for review 24 September 1990; revised manuscript received 8 March 1991*]