CHREV. 80

CHROMATOGRAPHY OF THE 1,4-BENZODIAZEPINES

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1. INTRODUCTION

     Since their introduction into clinical use in the early 1960s, the 1,4-benzo-diazepine drugs have established themselves as widely used tranquillizers, sleep inducers and muscle relaxants. With the extensive use of these compounds there has been a parallel evolution of analytical methods for their determination during development and in clinical and forensic situations. From the point of view of analysis in biological, especially human, samples, there are two important considerations. Firstly, the levels of the drugs are low, and secondly their chemically similar metabolites may also be pharmacologically active, and thus also require determination. These ana-

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lytical requirements have led to the widespread use of chromatographic techniques in the determination of the benzodiazepines. This review covers the progress in the chromatographic analysis of those benzodiazepines which are marketed or have had extensive clinical trials. It is likely that many more compounds of this type will come into clinical use in the future. The work reviewed here should provide a basis for further analytical work.
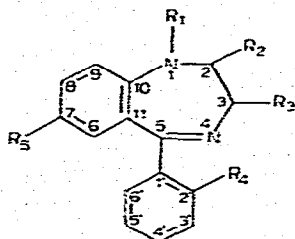

Fig. 1. General formula of the 1,4-benzodiazepines.

The general formula of the 1,4-benzodiazepines is shown in Fig. 1. The substituent at R_5 is invariably a halogen atom or a nitro group in the compounds considered here, an electronegative substituent in this position being essential for useful pharmacological activity[1]. The two main metabolic routes for the benzodiazepines involve dealkylation of the N_1 atom, and hydroxylation at C_3. In addition, the 7-nitro compounds are metabolized by reduction to the 7-amino- and 7-acetamidobenzo-diazepines. Hydroxylation of the 4'-position is an important process in several animals. The benzodiazepines are eliminated from the body mainly as the glucuronide and sulphate conjugates of the hydroxy and 7-amino metabolites. Metabolism of the benzodiazepines has been reviewed by Schwartz[2] and by Garrattini et al.[3].

Many benzodiazepines are hydrolyzed by strong acids to give benzophenones. These hydrolysis products have frequently been used in chromatographic analysis, and are listed in Table 1. It will be seen that certain benzophenones are formed by more than one benzodiazepine.

As is the case with other drugs, analytical support for the benzodiazepines is required in four areas:

(1) During development of the drugs, in elucidating their metabolism and studying their toxicology.

(2) In checking for purity and specification in the manufacture of the drug.

(3) In studying the pharmacokinetics and obtaining tissue and body fluid levels in clinical use.

(4) In obtaining tissue and body fluid levels in forensic situations, often following overdosage.

In the case of the benzodiazepines, chromatographic techniques have been widely used in all four areas. Each of these has its own requirements, and chromatographic methods developed for one field are not necessarily applicable in another. In the development of a drug, identification is often facilitated by the use of radio-actively labelled material, which is obviously not feasible in the other types of analysis. Chromatographic analysis of benzodiazepines for quality control and forensic

TABLE 1

HYDROLYSIS PRODUCTS OF SOME BENZODIAZEPINES

Benzodiazepine	Hydrolysis product	Abbreviation
Chlordiazepoxide	2-Amino-5-chlorobenzophenone	ACB
Desmethylchlordiazepoxide		
Demoxepam		
Desmethyldiazepam		
Oxazepam		
Chlorazepate		
Oxazolam		
Diazepam	2-Methylamino-5-chlorobenzophenone	MACB
3-Hydroxydiazepam		
Cloxazolam	2-Amino-7,2'-dichlorobenzophenone	ACCB
Nitrazepam	2-Amino-5-nitrobenzophenone	ANB
7-Aminonitrazepam	2,5-Diaminobenzophenone	DAB
7-Acetamidonitrazepam		
Clonazepam	2-Amino-2'-chloro-5-nitrobenzophenone	ANCB
7-Aminoclonazepam	2,5-Diamino-2'-chlorobenzophenone	DACB
7-Acetamidoclonazepam		
Bromazepam	2-Amino-5-bromobenzoylpyridine	ABBP
3-Hydroxybromazepam		
Flurazepam	2-Ethyldiethylamino-5-chloro-2'-fluorobenzophenone	
3-Hydroxyflurazepam		
N-Desalkylflurazepam	2-Amino-5-chloro-2'-fluorobenzophenone	ACFB
Flunitrazepam	2-Methylamino-5-nitro-2'-fluorobenzophenone	MANFB
Desmethylflunitrazepam	2-Amino-5-nitro-2'-fluorobenzophenone	ANFB

purposes involves detection of the drugs in relatively high concentrations, and methods used for these purposes are often inadequate for situations following therapeutic administration of the drugs. Blood levels of the benzodiazepines following therapeutic dosage are frequently in the range of 10–500 ng/ml. Chromatographic methods suitable for the drugs and their metabolites at this level in some cases require taking chromatographic techniques to their limits of sensitivity. This review emphasizes in particular the chromatographic analysis of the benzodiazepines in body fluids.

Extraction of the benzodiazepines from biological material has been described using a variety of solvent systems. The compounds have frequently been extracted from samples buffered to pH 9. As many benzodiazepines are strongly protein bound[4,5], vigorous shaking during the extraction step has been recommended by some workers[6]. When dealing with very low levels of the compounds, an acid clean-up stage to remove lipids and other interfering substances is often performed. Extraction solvents must be free of co-chromatographable impurities. In the case of urinary metabolites, chromatography of the intact compounds is normally carried out following hydrolysis of the conjugates by β-glucuronidase.

Gas–liquid chromatography (GLC) has been used extensively in the analysis of the benzodiazepines. It has often been possible to chromatograph the compounds intact, without derivatisation, especially at the microgram level. Chromatography at

low levels, such as those found in blood following a single therapeutic dose, is more demanding. At the nanogram level, adsorption processes become significant, especially for the N-desalkyl compounds, and processes such as photolytic decomposition may also be important. Partly for these reasons, a number of GLC methods involve chromatography of the benzophenone hydrolysis products rather than the benzodiazepines themselves. Determination of low levels of benzodiazepines or their hydrolysis products by GLC has often necessitated the use of electron capture detection. This in turn has implied injection of moisture free extracts onto the column, and use of clean-up procedures or selective extraction to minimize contamination of the detector[6,7].

Thin-layer chromatography (TLC) has been used for all of the benzodiazepines. Separations have most often been carried out on silica gel plates, often using solvent systems based on chloroform or ethyl acetate. Two-dimensional separations have been especially useful in metabolic studies. TLC of the hydrolysis products has frequently been used in toxicological and forensic work, sometimes resulting in reduced specificity. Detection is commonly achieved with modified Dragendorff reagent, platinum–iodine reagent, Bratton–Marshall derivatization in the case of compounds with primary amino groups, fluorescence quenching, and conversion to fluorophores (e.g., acridones) with concentrated acids.

2. CHROMATOGRAPHY OF THE BENZODIAZEPINES IN METABOLIC STUDIES AND CLINICAL PHARMACOLOGY

A. Diazepam, medazepam and their metabolites

(a) Diazepam

Diazepam and its metabolites have been studied more intensively than the other benzodiazepines. All three major metabolites (Fig. 2) have pharmacological activity. Oxazepam is marketed separately as a tranquilliser, and desmethyldiazepam (Ro 5-2180) and temazepam (Ro 5-5345) have both undergone clinical trials, and are likely to be made available for general medical use.

Gas chromatography has been used extensively in the determination of diazepam and metabolites in body fluids (Table 2), and the evolution of techniques from chromatography of the hydrolyzed products (benzophenones) to separation of the intact benzodiazepines reflects the introduction of improved materials for gas chromatography of low levels of compounds. It was quickly realised that these compounds were very suitable for electron capture gas chromatography, mainly because of the presence of the electronegative substituent at C_7. De Silva et al.[8], in the first gas chromatographic method to be published, hydrolyzed diazepam and its metabolites with 6 N hydrochloric acid to give the benzophenones ACB and MACB following extraction of plasma samples with diethyl ether. These derivatives were more volatile than the benzodiazepines and their chromatography was easier. A 2% Carbowax 20M stationary phase on silanized Gas-Chrom P was used in conjunction with a tritium electron capture detector (Fig. 3). Linear response to MACB and good reproducibility were obtained in a new column, but column performance deteriorated with age, and the average useful life was only 2 weeks. The method was later modified[9] by using a liquid phase of 2% Carbowax 20M–terephthalic acid[10].
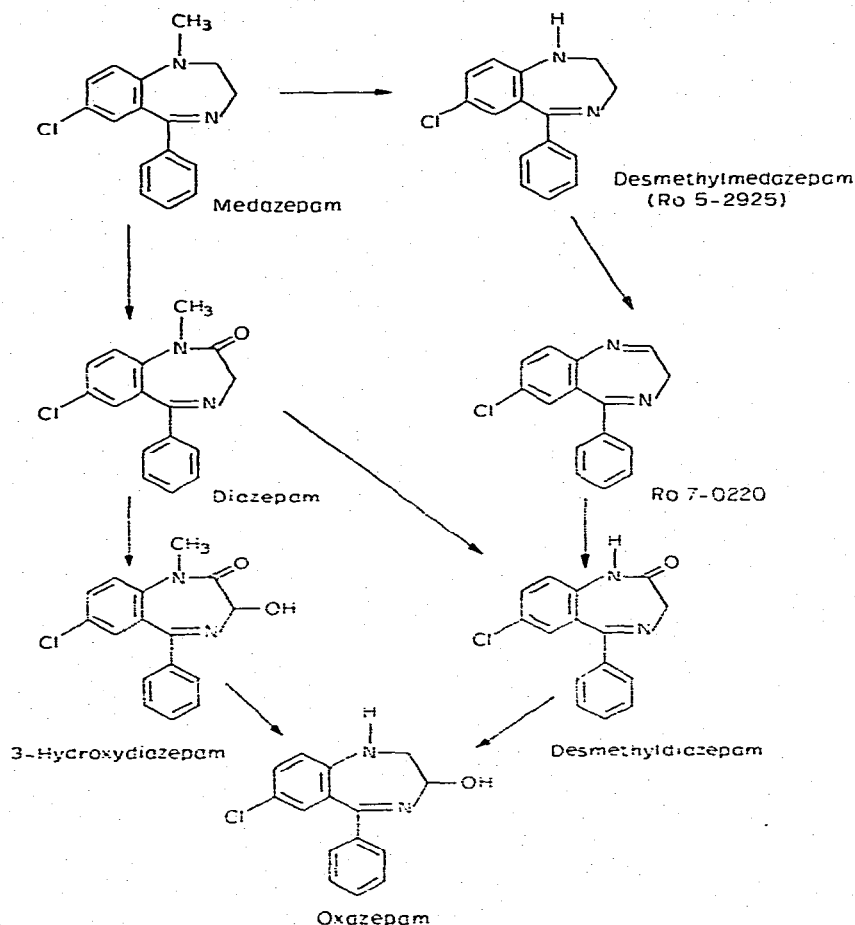
Fig. 2. Metabolic pathways for medazepam and diazepam.

This more polar phase had greater temperature stability. better coating characteristics and longer column life.

A very similar method was subsequently published by Cano et al.[11,12]. All these methods based on chromatography of the benzophenones suffered from a lack of specificity. as hydrolysis of diazepam and its three metabolites gives rise to only two benzophenones. It is therefore not possible to differentiate 3-hydroxydiazepam from diazepam. or oxazepam from desmethyldiazepam. In practice. this may not be a major shortcoming in the analysis of blood samples. as only diazepam and its desmethyl metabolite are present in significant amounts in the circulation following a single therapeutic dose or short-term administration. In addition. there were problems regarding co-extracted compounds which gave peaks at or close to the retention volumes of the benzophenones. This point was considered in some detail in De Silva et al.'s original paper[8]. Washing the acid phase with ether prior to hydrolysis gave cleaner chromatograms than clean-up of ether extracts or acid with various

TABLE 2

GLC METHODS FOR DIAZEPAM, MEDAZEPAM AND THEIR METABOLITES

Workers	Column	Column temperature (°C)	Detector	Internal standard	Extraction solvent	Remarks
De Silva et al.[8] (1964)	2-ft, stainless steel, 2% Carbowax 20M	190	Tritium ECD	—	Diethyl ether	Hydrolysis to ACB and MACB prior to chromatography
De Silva et al.[9] (1966)	2-ft, stainless steel, 2% Carbowax 20M-TPA on Gas-Chrom P, 100–120 mesh	215	Tritium ECD	—	Diethyl ether	As above
Cano et al.[11] (1967)	2-m stainless steel, 2% Carbowax 20M on Chromosorb	195	Tritium ECD	—	Diethyl ether	As above
Marcucci et al.[13] (1968)	2-m glass, 3% OV-1 on Gas-Chrom Q, 60–80 mesh	245	FID	BACB	Diethyl ether	Intact benzodiazepines, relatively high concentrations
De Silva and Puglisi[a] (1970)	4-ft, glass, 3% OV-17 on Gas-Chrom Q, 60–80 mesh	235	⁶³Ni ECD	Medazepam	Diethyl ether, followed by acid clean-up	Method for both intact diazepam and medazepam. No internal standard when used for medazepam
Foster and Frings[19] (1970)	2-m × 2-mm I.D. glass, 3% SE-30 on Chromosorb W, 80–100 mesh	205	FID	—	Chloroform	Detection of metabolites not reported. Suitable for overdose or chronic medication situations
Van der Kleijn et al.[15] (1971)	1-m glass, 3% OV-17 on Gas-Chrom Q, 80–100 mesh	235	⁶³Ni ECD	Griseofulvin or medazepam	Diethyl ether	

| Reference	Column	Temp.	Detector	Internal standard	Extraction	Comments
Berlin et al.[30] (1972)	6-ft. × 3-mm glass, 3% OV-17 on 60-80 mesh Gas-Chrom Q	240	63Ni ECD	Griseofulvin	Benzene	Used for bioavailability studies on diazepam. Intact drug
Zingales[7] (1973)	120-cm × 2-mm I.D. glass, 2% OV-17 on Chromosorb G, 80-100 mesh	235	63Ni ECD	—	Toluene–heptane–isoamyl alcohol. Acid clean-up for low concentrations	Intact diazepam and metabolites
Baird et al.[29] (1973)	3-ft. × ¼-in. glass, 3% OV-225 on Gas-Chrom Q, 80-100 mesh	235	63Ni ECD	Prazepam	Diethyl ether with acid clean-up	Suitable for medazepam diazepam and metabolites intact
Mallach et al.[38] (1973)	150-cm × 3-mm I.D. glass, 3% OV-25 on Chromosorb W AW DMCS, 80-100 mesh	210-240 (10/min)	Thermionic (NFID)	MACB	Diethyl ether, acid clean-up	Improved sensitivity to medazepam and desmethylmedazepam. Temperature programme
Howard et al.[21] (1974)	1-ft. × 4-mm I.D. glass, 3% OV-225 on Gas-Chrom Q, 60-80 mesh	235	63Ni ECD	Prazepam	Ethyl benzoate	Rapid extraction method for toxicology, unsuitable for low concentrations
Knowles and Ruelius[17] (1972)	2-ft. × 2-mm stainless steel, 2% XE-60 on Chromosorb W, 80-100 mesh	240	63Ni ECD	—	Diethyl ether and acid clean-up	Method for oxazepam. Chromatography of ACB
Vessman et al.[49] (1972)	6% OV-17	235	Tritium ECD	Lorazepam	Methylene chloride and acid clean-up	Hydrolysis to benzophenones
Belvedere et al.[50] (1972)	2-m × 4-mm I.D. glass, 3% OV-17 on Chromosorb Q, 100-120 mesh	260	63Ni ECD	Diazepam	Diethyl ether	Method for temazepam using TMS derivative
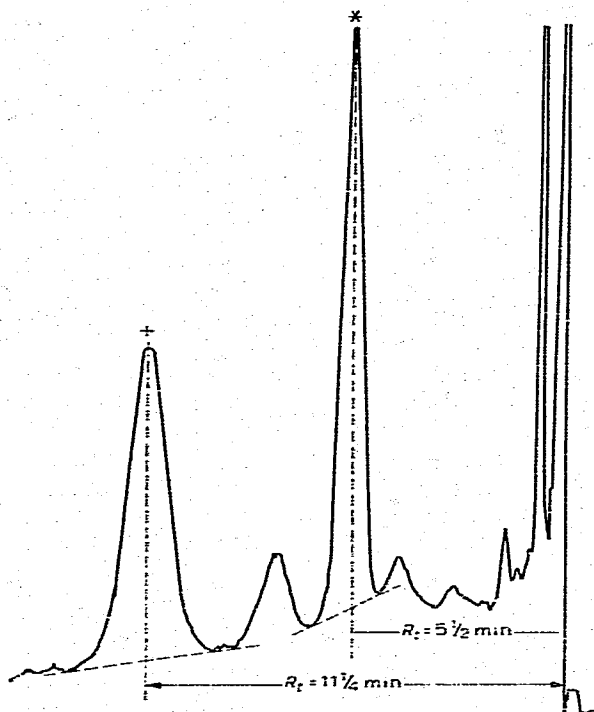
Fig. 3. Chromatogram of diazepam and its N-demethylated metabolite in blood determined as the MACB and ACB compounds, respectively. (Reproduced from ref. 8 with permission of the publishers.)

adsorbents. Although good reproducibility was obtained, internal standards were not used in this early work.

In 1968, Marcucci et al.[13] reported the chromatography of the intact benzodiazepines using an OV-1 stationary phase. Flame ionization detection was used in most of the work, with electron capture detection to improve sensitivity in several experiments. The compound 2-N-benzylamino-5-chlorobenzophenone (BACB) was used as an internal standard for the gas chromatography, eluting after the benzodiazepines. Interfering peaks from co-extracted material were not encountered in work with rat blood samples. This work was later presented in greater detail[14]. The method was modified by Van der Kleijn et al.[15] to incorporate an OV-17 stationary phase. BACB was again used as the internal standard. The higher temperatures required to elute the benzodiazepines intact necessitated the use of the more stable silicone stationary phases, and high-temperature electron capture detectors incorporating a ^{63}Ni source.

De Silva and Puglisi[6] used an OV-17 phase and a ^{63}Ni electron capture detector for analysis of medazepam, diazepam and their metabolites (Fig. 4). The method incorporated a clean-up procedure to remove lipids and other endogenous co-extracted material. In the diazepam assay, medazepam was used as an internal standard, being taken through the entire extraction and clean-up procedure. Ether extraction was again used, giving recoveries of $86 \pm 6.0\%$ for diazepam and $94 \pm 6.0\%$ for its
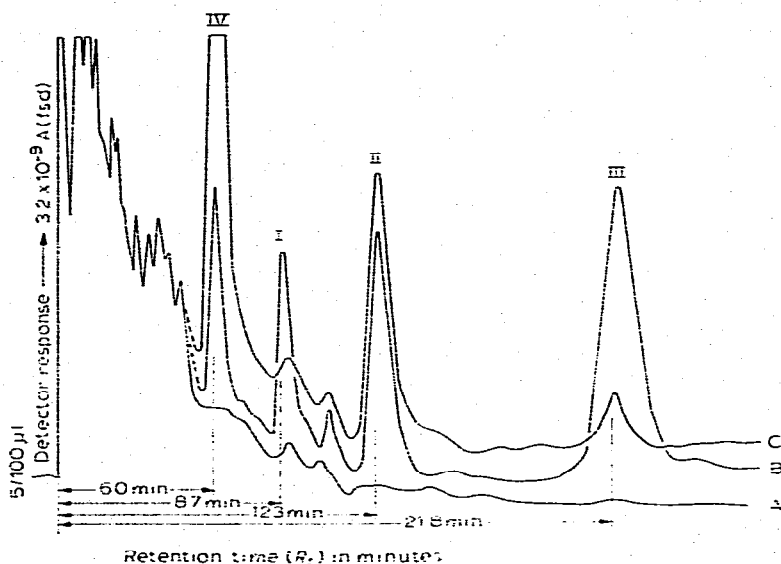
Fig. 4. Gas chromatograms of diethyl ether extracts of (A) patient control urine, (B) control urine containing added authentic standards, and (C) patient urine (0–24 h) post-medication with diazepam: 3%, OV-17 stationary phase. I, Diazepam; II, N-desmethyldiazepam; III, 3-hydroxydiazepam; IV, oxazepam. (Reproduced from ref. 6 with permission of the publishers.)

desmethyl metabolite. In urine assays, 80–85% recoveries were reported for oxazepam and 3-hydroxydiazepam. Sensitivity was in the range 0.01–0.04 μg/ml in blood using a 1-ml sample, and could be improved by increasing the sample size.

De Silva and Puglisi[6] discussed a number of factors relevant to gas chromatography with electron capture detection. Concentration of hydrochloric acid used in the back-extraction of ether is critical to benzodiazepine stability. The hydroxy derivatives require the use of 6 N hydrochloric acid for quantitative back-extraction, this being verified by TLC studies. In contrast to the earlier work of Marcucci et al.[13], OV-1 was not found to be a suitable phase, as resolution of the various compounds from a biological extract was incomplete, resulting in overlapping peaks. Under the conditions used in this assay, hydroxydiazepam had an inconveniently long retention time of 22 min, and a relatively low detector response. The situation could be improved by formation of the trimethylsilyl derivative using hexamethyldisilazane/trimethylchlorosilane reagent. A shortened retention time (12.8 min) was obtained with an approximately 10-fold increase in sensitivity. Overlap with the desmethyldiazepam peak was overcome by a differential extraction technique.

The use of a benzodiazepine internal standard, which could be taken through the entire assay, represented a useful advance. However, the use of medazepam, as reported by De Silva and Puglisi, suffers from some disadvantages. The compound is susceptible to decomposition when stored in solution, has a short retention time, giving possible overlap with impurity peaks, and also a lower electron capture response than diazepam and its metabolites. In addition, back-extraction into hydrochloric acid more concentrated than 2 N causes partial conversion of medazepam into a quinoxaline.

There has been widespread use of methods based on those of Marcucci *et al.* and De Silva and Puglisi. Modifications have included minor changes in the chromatography and extraction procedures[16,17], and the use of different internal standards[18].

The method of De Silva and Puglisi is fairly long, because of the clean-up procedure involved. Several groups have recently published methods for the determination of diazepam which involve the use of more rapid extraction procedures. These methods have produced useful results, but in most cases have been used to monitor diazepam levels after overdosage or chronic administration. Quantitation of very low levels of diazepam and its metabolites using electron capture detection will require an acid clean-up stage, as interference due to co-extracted endogenous compounds becomes significant at low levels.

Foster and Frings[19] published a rapid method in which plasma was extracted with chloroform and an aliquot injected on to the chromatograph. Using a flame ionization detector (FID), the method was suitable for toxicological work with high levels of the drug. Chromatography of the metabolites was not reported. Berlin *et al.*[20] used a benzene extraction without clean-up in the determination of bioavailability of diazepam in various formulations. The internal standard was griseofulvin and was added to the sample extract immediately prior to chromatography. Diazepam and its desmethyl metabolite were determined in plasma at steady-state concentrations. Detection limits of 30 and 40 ng/ml, respectively, were quoted using electron capture detection.

Zingales[7] has studied steady-state levels of diazepam and metabolites in plasma, erythrocytes and urine using electron capture GC with an OV-17 phase. The extraction solvent consisted of toluene–heptane–isoamyl alcohol (80:20:1.6). The main purpose of the alcohol was to prevent adsorption of the benzodiazepines on to glass. This extraction mixture was claimed to give cleaner chromatograms than those produced following extraction with ether or chloroform. The method also included a selective extraction procedure whereby each compound could be isolated for further characterization Extraction experiments showed that the toluene–heptane ratio was critical for quantitative extraction of the hydroxy metabolites (Fig. 5). Zingales[7] also gave data for variation of partition ratios of the benzodiazepines as a function of buffer pH and of acid concentration. It was shown that the optimum buffer pH for extraction was between 8.5 and 10, and that 6 *N* hydrochloric acid was required to completely remove the hydroxy metabolites from the extraction solvent. This last result is similar to that obtained by De Silva and Puglisi. Extraction without clean-up was used for routine clinical and toxicological work with acid clean-up being reserved for determinations of very low levels of diazepam and its metabolites. An internal standard was not used in this work.

A rapid extraction method for toxicological work has recently been developed by Howard *et al.*[21]. This followed a procedure for amphetamines described by Ramsey and Campbell[22], but used ethyl benzoate instead of chloroform to permit use of direct injection of the extraction solvent and electron capture detection. Satisfactory results were obtained for diazepam and all its metabolites, with detection limits of 0.02–0.1 *μ*g/ml. The method was not considered suitable for determination of very low levels of the compounds.

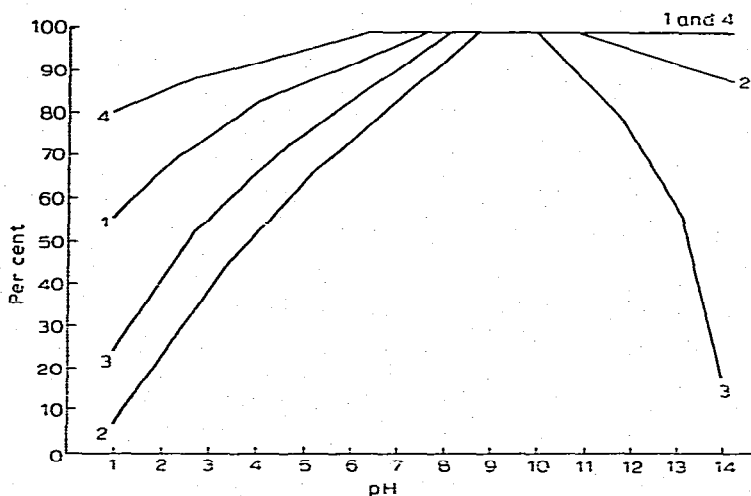Separation of diazepam and its metabolites by high-pressure liquid chro-

Fig. 5. Partition characteristics of diazepam (1), desmethyldiazepam (2), oxazepam (3) and 3-hydroxydiazepam (4) between toluene–*n*-heptane–isoamyl alcohol and various buffer solutions. (Reproduced from ref. 7 with permission of the publishers.)

matography (HPLC) was reported by Scott and Bommer[23]. Liquid–solid chromatography was carried out using Durapak (OPN), 36–75 μm. in a 100 cm × 1 mm column with hexane–isopropanol (95:5) as a solvent at a flow-rate of 1.0 ml/min. A UV monitor set at 254 nm was used as detector. Complete resolution of diazepam and its three major metabolites was achieved at a sensitivity of about 2 μg per compound.

Schwartz and co-workers[24,25] used TLC to follow the metabolism of diazepam labelled with ^{3}H in the 5-phenyl ring following administration to dogs and humans. Bands were detected by fluorescence quenching under UV light, and the bands scraped off for spectroscopic characterization. This approach has been adopted for other benzodiazepines during development of the drugs. In the analysis of extracts of biological materials. De Silva *et al.*[9] used a two-dimensional development on silica gel plates, compounds being rendered visible by immersion of the plate in iodine vapour. Ruelius *et al.*[26] also used TLC to separate diazepam from its metabolic products. Jommi *et al.*[27,28] studied diazepam metabolism in rabbits using TLC methods and column chromatography with magnesium silicate and alumina. Further details of the TLC separation of diazepam and its metabolites are given in Table 3.

(b) Medazepam

Medazepam differs from diazepam in having no carbonyl group at C_2. Its main metabolites are shown in Fig. 2, and include diazepam and its biotransformation products. Chromatographic methods for the determination of medazepam are therefore linked to those described for diazepam.

The method of De Silva and Puglisi[6] was the first gas chromatographic assay for medazepam and metabolites in biological samples, and has already been referred to in connection with the assay of diazepam. No internal standard was used. The electron capture detector does not respond well to either medazepam or its desmethyl

TABLE 3

TYPICAL TLC SYSTEMS FOR BENZODIAZEPINES

Compound	Reference	Solvent system	Detection	Use
Diazepam	Schwartz et al.[24]	(1) Heptane–chloroform–ethanol (10:10:1)	Iodine vapour and scintillation counting	Metabolic studies using ^{3}H-labelled material
		(2) Heptane–chloroform–acetic acid–ethanol (5:5:1:0,3) Or isopropanol–concentrated ammonia (20:1)		Blood level distribution studies
	De Silva et al.[9]	Chloroform–acetone (90:10)	UV	
	Beckstead and Smith[117]	Chloroform–toluene–methanol (10:9:1)	UV, modified Dragendorff, or chlorine–toluidine	Separation from intermediates and impurities
Medazepam	Schwartz and Carbone[31]	As for diazepam TLC[24] Also heptane–ethyl acetate–ethanol–conc. ammonia (5:5:1:0,3)	UV and scintillation counting	Metabolic studies on ^{14}C-labelled material
	Lauffer and Schmid[36]	Cyclohexane–diethylamine–benzene (80:15:5)	UV, fluorimetry after treatment with phosphoric acid	Plasma level analysis
Oxazepam	Sisenwine et al.[39]	(1) Chloroform–ethanol–acetone (8:1:1) (2) Ethyl acetate–ethanol–ammonia (5:5:1)	UV, Bratton–Marshall	Metabolic studies in animals and man
	Steidinger and Schmid[41]	Toluene–tetrabutylamine–methanol (8:1:1) Or ethyl acetate–dichloromethane (2:5)	Fluorimetry	Metabolic studies in man
	Beckstead and Smith[117]	Toluene–nitromethane–methanol (11:8:1)	UV, Dragendorff	Separation from intermediates and impurities

Chlordiazepoxide	Schwartz and Postma[53]	(1) Chloroform–ethanol (9:1) (2) Ethyl acetate–ethanol (95:5)	UV, scintillation counting	Metabolic studies in rat using ^{14}C-labelled drug
	Beckstead and Smith[117]	Chloroform–methanol (10:1)	UV, modified Dragendorff, chlorine–o-toluidine	Separation of chlordiazepoxide hydrochloride from impurities and intermediates
Bromazepam	De Silva and Kaplan[70]	Heptane–chloroform–ethanol (10:10:1) Or ethyl acetate	UV, scintillation counting, GLC	Blood level analysis
	Sawada[71]	Chloroform–acetone (9:1)	Bratton–Marshall, Dragendorff	Metabolic studies in animals
Lorazepam	Schillings et al.[82]	Chloroform–ethanol–acetone (8:1:1) Or ethyl acetate–ethanol–ammonia (5:5:1)	UV, Bratton–Marshall	Metabolic studies
Nitrazepam	Rieder[88]	Ethyl acetate–n-propanol–diethylamine (70:30:1) Toluene–acetone–ammonia (50:50:1)	UV, Folin's reagent, Bratton–Marshall	Metabolic studies and forensic analysis
	Beckstead and Smith[117]	Chloroform–methanol (10:1) Or chloroform–toluene–methanol (10:9:1)	UV, Bratton–Marshall, chlorine–o-toluidine	Separation from impurities and intermediates
Flurazepam	Schwartz and Postma[105]	Ethyl acetate–ethanol–ammonia (95:5:0.5) Benzene–ethyl acetate–ethanol–ammonia (80:20:10:0.2)	UV, scintillation counting	Metabolic studies
Clonazepam	De Silva and Puglisi[104]	n-Propanol–benzene–conc. ammonia (80:20:1)	Pulse polarography after UV	Analysis of major urinary metabolites
	Eschenhof[100]	(1) Toluene–acetone–ammonia (50:50:1) (2) Benzene–n-propanol–ammonia (80:20:1)	UV, Bratton–Marshall, scintillation counting	Metabolic studies

metabolite (Ro 5-2925), although both compounds are eluted intact from the chromatographic column. The loss of the carbonyl function at position 2 evidently significantly decreases the electron capturing ability of the benzodiazepine system. Following 30-mg doses of the drugs to humans, De Silva and Puglisi were unable to detect desmethylmedazepam in the blood after a single dose (detection limits for this compound and medazepam being about 0.05 μg/ml), while diazepam and the major blood metabolite desmethyldiazepam were readily detected. An alternative procedure for electron capture gas chromatography has been reported by Baird et al.[29], in which a more polar silicone phase (OV-225) was used to increase the resolution between diazepam and desmethyldiazepam. This made it possible to use the benzodiazepine prazepam as an internal standard for the assay, this compound eluting between diazepam and desmethyldiazepam (Fig. 6). As in the previous method, sensitivity to medazepam and desmethyldiazepam was low, and the latter could not be detected in the blood following a single therapeutic dose of medazepam (10 mg, orally). The method also has application in the analysis of diazepam, but has the disadvantage that 3-hydroxydiazepam has a high retention volume unless derivatized. In both electron capture gas chromatographic methods, acid clean-up of the extracts is con-
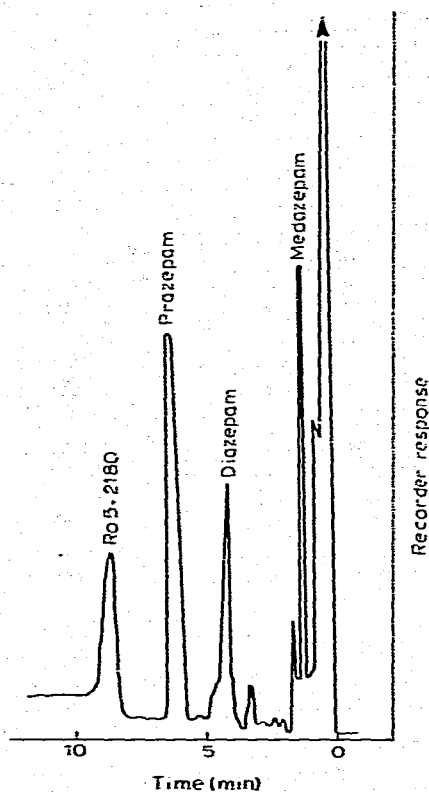

Fig. 6. Gas chromatogram of medazepam and its major metabolites after extraction from plasma. Prazepam internal standard, OV-225 stationary phase. (Reproduced from ref. 29 with permission of the publishers.)

sidered essential, as medazepam's relatively low response and proximity to the solvent front make it necessary to eliminate as far as possible endogenous compounds which would co-chromatograph.

Some of the disadvantages inherent in electron capture chromatography of medazepam have been overcome by Mallach et al.[30] by use of a nitrogen-selective (thermionic) detector. Using a 3% OV-25 stationary phase, medazepam and its major benzodiazepine metabolites were resolved by use of a temperature programme from 210° to 240° at 10°/min. The benzophenone MACB was used as an internal standard for quantitative chromatography. Silylation of 3-hydroxydiazepam by the method of De Silva and Puglisi[6] shortened its retention time and again increased its sensitivity. An ether extraction was used with an acid clean-up using hydrochloric acid of two concentrations to take account of the acid instability of medazepam and the difficulty of extraction of the hydroxy metabolites. The method was used successfully in the analysis of medazepam and metabolites in human serum and urine following therapeutic dosage, although large sample volumes of sera (4 ml) were used. Compared with electron capture detection, the thermionic detector provided a significant improvement in sensitivity for medazepam and desmethyl-medazepam. Use of a temperature programme greatly improved the resolution of metabolites, although it was not stated whether the system used could also resolve the major urinary metabolite 2-amino-3-benzoyl-5-chlorophenol reported by Schwartz and Carbone[31].

Improved sensitivity to medazepam and desmethylmedazepam has also been achieved by Hailey et al.[32] using a Coulson detector. No co-extracted material was detectable in the analysis of spiked rat blood samples, and using an OV-17 stationary phase, temperature programming was successfully used to increase resolution. The OV-225 phase used in previous work[21] was unsuitable for temperature programming with this detector owing to the high bleed-off of nitrogenous material.

The use of temperature programming seems especially suitable in the analysis of medazepam and its metabolites by GLC. Even with isothermal operation, however, resolution of medazepam from the solvent front can be improved by use of a non-silicone stationary phase, and Howard[33] used the polyimide phase Poly I-110 to increase the relative retention time of medazepam from that obtained on OV-225.

TLC separation of [14C]medazepam and its metabolites has been used by Rieder and Rentsch[34] and by Schwartz and Carbone[31] in studies on the metabolism of the compound in several species. Two-dimensional TLC on silica gel plates was carried out in similar fashion to that described by Schwartz and co-workers for diazepam metabolites.

Besserer et al.[35] reported the determination of medazepam by TLC following ether extraction of serum. Lauffer and Schmid[36] used spectrofluorimetry to quantitate medazepam following separation by TLC. The substances were located by fluorescence quenching under UV light (254 nm), and also after spraying the plate with various reagents. Fluorescence spectra were measured on plate (using a scanner) and in solution. Strong fluorescence was achieved by pre-treating the plate with 0.1 N hydrochloric acid and then spraying it with orthophosphoric acid. The method was used to measure medazepam in plasma and gastric juice and was used to monitor the blood levels in humans following a therapeutic dose.

(c) Oxazepam

The diazepam metabolite oxazepam has been marketed as a tranquilliser for some years. It differs from diazepam and non-hydroxylated benzodiazepines in that a substantial proportion of the drug in the bloodstream is present as the glucuronide[37].

As with other benzodiazepines, metabolic studies were carried out with the aid of the ^{14}C-labelled compound, and Walkenstein et al.[38] used ascending paper chromatography to separate urinary metabolites from various animal species. Separation of oxazepam and its glucuronide was achieved, and it was noted that rat urine contained several other metabolites, although they were not identified. The metabolism was further investigated by Sisenwine et al.[39] using a two-dimensional TLC system on silica gel F-254 plates. As well as the use of analytical TLC, metabolites were identified by NMR and mass spectrometry after separations on a preparative scale. Metabolites identified included benzodiazepines with phenolic or methoxy substituents on the 5-phenyl ring, and also a number of open-chain compounds. It was suggested that oxazepam might be in equilibrium with the tautomeric opened ring.

Weist[40] has described a TLC system for oxazepam which can be used as a preparation for subsequent quantitative determination by fluorimetry. The drug was detected on plate after heating with 70% perchloric acid, and the method was used in the analysis of oxazepam in various body fluids. Steidinger and Schmid[41] used a thin-layer scanner to measure urinary oxazepam. Urine samples were incubated with β-glucuronidase, extracted with dichloromethane, and the dried residue was chromatographed on silica gel. Fluorimetric assay was carried out on plate following treatment with trichloroacetic acid or by eluting oxazepam from the plate and determining it separately by spectrofluorimetry.

Kamm and Kelm[42] used TLC of the hydrolysis product (ACB) followed by diazo coupling with an azo dye and spectrophotometry to measure oxazepam blood levels. Sunjic et al.[43] used TLC and column chromatography to separate the diastereoisomers of oxazepam-camphanic acid esters. Hydrolysis of the esters resulted in a racemate.

Gas chromatography has also been widely used to determine oxazepam. When diazepam and its metabolites are gas chromatographed, it is observed that oxazepam is eluted first, despite being the most polar compound. This chromatographic behaviour is due to thermal decomposition. Oxazepam rapidly loses a molecule of water on column, forming 6-chloro-4-phenylquinazoline-2-carboxaldehyde (Fig. 7). This compound is more volatile than diazepam and its other two major metabolites, which are eluted intact. The decomposition process was investigated by Sadee and Van der Kleijn[44] and by Forgione et al.[45] using GLC–MS and direct mass spectrometry. The mechanism of dehydration was demonstrated by use of [18]O- or [2]H-labelled oxazepam. Conversion of oxazepam into its decomposition product was almost 100%. Medazepam, desmethylmedazepam, diazepam and desmethyldiazepam were eluted intact, but 3-hydroxydiazepam gave a smaller response than expected with total ion current detection, suggesting partial decomposition. As the decomposition of oxazepam is rapid and almost quantitative, quantitation without hydrolysis to ACB is still possible. Preparation of the trimethylsilyl derivative of oxazepam gives a peak with a retention time similar to that of the decomposition product[6]. More
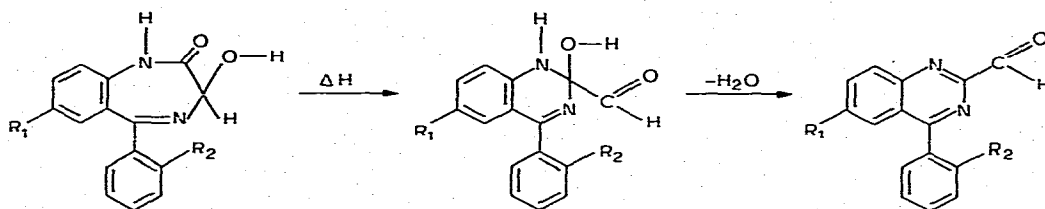
Fig. 7. Thermal decomposition of N-desalkyl-3-hydroxybenzodiazepin-2-ones.

recently, Frigerio *et al.*[16] have shown that similar decompositions occur for lorazepam and 3-hydroxynitrazepam. GC–MS again being used. Similar results have been obtained with 3-hydroxybromazepam and 3-hydroxydesalkylflurazepam[17] and would appear to be general for the N-desalkyl-3-hydroxybenzodiazepines.

Knowles and Ruelius[37] reported an electron capture gas chromatographic method for determining the drug in biological fluids which has been used to measure blood levels following therapeutic dosage. The method was a modification of that of De Silva *et al.*[9]. Oxazepam was extracted from phosphate-buffered serum with ether and back-extracted into 12 N sulphuric acid. The acid layer was washed with ether and then heated to 100° for 1 h to hydrolyze oxazepam to the benzophenone ACB, which was chromatographed on XE-60 stationary phase. An external standard technique was used to check the instrument performance. The oxazepam glucuronide in blood was determined in a similar manner after incubation with β-glucuronidase. This hydrolysis method was preferred to chromatography of the intact compound as cleaner chromatograms and higher sensitivity were obtained. A sensitivity limit of 20 ng/ml was quoted, compared with 50 ng/ml for the intact compound mentioned by Marcucci *et al.*[18]. Use of the hydrolysis method implied, as usual, some loss of specificity, but this is less of a problem in the case of oxazepam compared with most other marketed benzodiazepines, as metabolites other than the oxazepam glucuronide are present only in very small amounts.

Vessman *et al.*[19] have also published a GLC method for oxazepam and its glucuronide based on electron capture detection of ACB. Free oxazepam was extracted from serum buffered at pH 7.4 with methylene chloride containing lorazepam as internal standard. The benzodiazepines were back-extracted into sulphuric acid and hydrolyzed before re-extraction and chromatography. Quantitative determinations down to 1 ng/ml were performed. This work included a discussion on optimum extraction and hydrolysis conditions.

(d) Temazepam

3-Hydroxydiazepam (temazepam) has been used in a number of clinical studies, and a gas chromatographic method was reported by Belvedere *et al.*[50]. A similar procedure to that mentioned by De Silva and Puglisi[6] was used, temazepam being silylated at the hydroxy group before chromatography on 3 % OV-17. Diazepam was used as an internal standard. Like De Silva and Puglisi, the authors reported an increase in sensitivity to electron capture detection following silylation.

B. Chlordiazepoxide

Chlordiazepoxide was the first of the benzodiazepines to be marketed, and its metabolic pathways (Fig. 8) were established by use of TLC separation in conjunction with the radioactively labelled drug. Thus, Koechlin and co-workers[51,52] used TLC and paper chromatography with [2-14C]chlordiazepoxide to study the metabolism of the compound in man and dog, and established that demoxepam was a plasma metabolite in both species. Quantitation was achieved using a chromatogram scanner, by scintillation counting after elution of the spots, or by hydrolysis followed by a Bratton–Marshall reaction. Ethyl acetate-extractable urinary metabolites were isolated in milligram amounts using a silica gel column. A subsequent radio-TLC study by Schwartz and Postma[53] showed the presence of a third metabolite, desmethylchlordiazepoxide, in man. A further metabolic study in the rat[54] used two-dimensional TLC with reference compounds and high-resolution mass spectrometry to establish the identity of the urinary metabolites. Pribilla[55] developed TLC systems for chlordiazepoxide and its metabolites and applied them to the analysis of urine, blood and tissue samples. A large number of solvent systems and detection methods were tested and compared. The most reliable method involved hydrolysis to the benzophenone ACB followed, if required, by diazotisation and coupling.

Urinary chlordiazepoxide metabolites were also studied by Kimmel and Walkenstein[56] using TLC and paper chromatography of 14C-labelled material
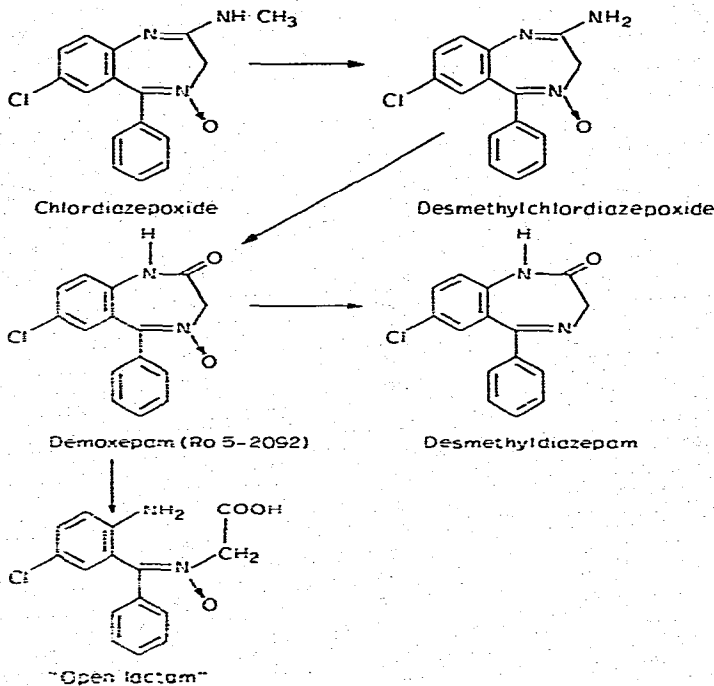

Fig. 8. Metabolic pathways for chlordiazepoxide.

followed by autoradiography and scintillation counting. Kaplan et al.[57], in a pharmacokinetic study in the dog, used TLC for urine samples to eliminate interfering fluorescence. Miachon and Revol[58] used TLC and paper chromatography in their study on chlordiazepoxide metabolism in rabbits, with fluorimetric and Bratton–Marshall detection.

Schwartz et al.[59] have used two-dimensional TLC with NMR and mass spectroscopy in studying the metabolites of [2-^{14}C]demoxepam in the dog. Several metabolites were identified in the dog with phenolic groups in either benzenoid ring. In man[60], demoxepam was the major urinary metabolite with small amounts of oxazepam and the 9- and 4-hydroxy-N-oxides. Desmethyldiazepam was the only faecal metabolite identified. Spots were located under UV light and then removed and quantitated by scintillation counting.

Brooks et al.[61] have reported a toxicological assay for chlordiazepoxide and its desmethyl and lactam metabolites using pulse polarography to determine the compounds after separation on silica gel. The sensitivity of the assay is about 0.5 μg/ml.

The identification and determination of desmethyldiazepam in human plasma following chronic administration of chlordiazepoxide has been reported by Dixon et al.[62] using a combination of TLC and electron capture GC. The results obtained gave good agreement with those found using a radioimmunoassay technique that was specific for desmethyldiazepam in the presence of chlordiazepoxide and its other metabolites.

Analytical HPLC of chlordiazepoxide was reported by Scott and Bommer[23] in their study on the separation of the benzodiazepines from biological material. Using a UV detector, a sensitivity in the microgram range was reported.

The first gas chromatographic method for chlordiazepoxide was described by De Silva[63], and involved ether extraction of the compound from blood, followed by hydrolysis to ACB prior to chromatography. This method obviously did not distinguish between the drug and its metabolites. Martin and Street[64] chromatographed chlordiazepoxide without prior hydrolysis using heat-treated (350°) SE-30 on silanized Chromosorb W and a stainless-steel column at a temperature of 245°. However, under these conditions two peaks were obtained, indicating possible decomposition on the column. Possibly as a result of the difficulties with GLC analysis of chlordiazepoxide, the most widely used method for the drug and its metabolites in body fluids in recent years has involved spectrofluorimetry[53,65]. However, in 1971, Zingales[66] reported the successful gas chromatography of intact chlordiazepoxide using electron capture detection. In this method, the drug was extracted with n-heptane containing 1.5 % isoamyl alcohol from plasma buffered to pH 9. A recovery of about 90 % was achieved. At high concentrations of the drug, a portion of the extraction liquid was injected directly on to the chromatographic column. With low plasma levels, the drug was back-extracted into 0.1 N hydrochloric acid before re-extraction and concentration. Higher recoveries (97–100 %) were obtained at pH 7.4, but the higher pH value was chosen because the parent drug is selectively extracted under these conditions. Analyses were carried out using a 4 ft. × ¼ in. O.D. column packed with 2 % OV-17 on 80–100 mesh Chromosorb W, at an oven temperature of 275°. This different chromatographic behaviour presumably reflected the use of improved packing materials and more careful column treatment.

Although nitrones such as demoxepam can be chromatographed by GLC, the results are not satisfactory from the point of view of quantitation, as compounds of this type were found by Sadee and Van der Kleijn[34] to partially decompose on the column by loss of oxygen from the N_1 position.

C. Chlorazepate

Chlorazepate incorporates a carboxylic acid function in the diazepine ring (Fig. 9). In acid solution, it is quickly converted into desmethyldiazepam[67]. The main metabolic products are desmethyldiazepam and oxazepam. Gros and Raveux[68] reported the TLC of chlorazepate on silica gel G using as solvent n-butanol–methanol–formamide (70:15:5). At 4°, R_F values of 0.20 and 0.80 were obtained for the drug and desmethyldiazepam, respectively, and the spots were made visible by fluorescence quenching.
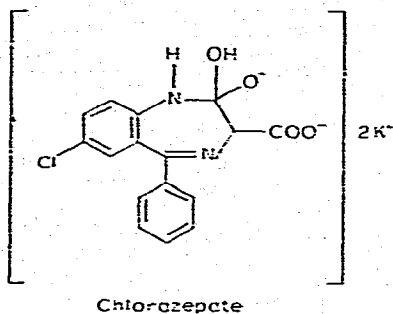

Chlorazepate

Fig. 9. Chlorazepate.

Analysis of chlorazepate and its metabolites in blood and urine was described by Viala et al.[69]. The metabolites are ether extracted from blood buffered to pH 9, and processed for GLC analysis as described above under *Diazepam*. Chlorazepate is retained in the aqueous phase, which is then adjusted to pH 3 and heated at 40° for 5 min. Under these conditions, chlorazepate is converted to desmethyldiazepam, which is ether extracted after readjustment of the aqueous phase to pH 9, and chromatographed in the usual way.

D. Bromazepam

Bromazepam has recently been marketed in Europe as a hypnotic, and differs from other benzodiazepines in having a 7-bromo substituent and a 5-pyridyl rather than a 5-benzyl ring (Fig. 10). TLC and electron capture gas chromatography of this compound have been described by De Silva and Kaplan[70]. TLC was carried out on silica gel using either ethyl acetate or n-heptane–chloroform–ethanol as solvents, and fluorescence quenching was employed for rendering the spots visible. Sawada and co-workers[71,72] used TLC to study urinary metabolites of bromazepam, with standard detection techniques and additional spectroscopic characterization.
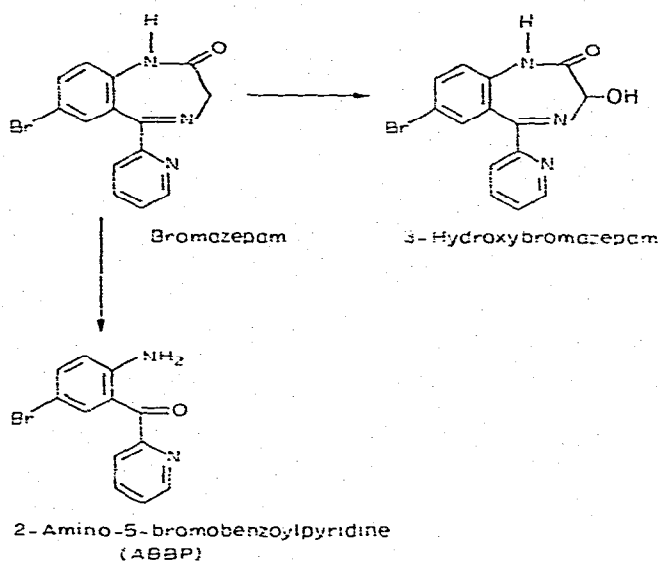
Fig. 10. Bromazepam and metabolites.

Preliminary GLC studies by De Silva and Kaplan[70] showed that intact bromazepam was thermally unstable at the temperatures required for elution. Analysis was therefore carried out by hydrolysis with 6 N sulphuric acid to give 2-amino-5-bromobenzoylpyridine (ABBP), which was chromatographed on a 2-ft. stainless-steel column containing 2% Carbowax 20M–TPA. No internal standard was used, and a minimum detectable level of 5.0 ng was reported. The overall recovery from blood was 61 ± 3%. This low recovery was attributed in part to further hydrolysis of ABBP to p-bromoaniline and nicotinic acid.

Greaves[73] has successfully chromatographed bromazepam intact at the microgram level, using flame ionization detection and an OV-17 stationary phase. Treatment of the compound with BSTFA apparently produced a suitable silyl derivative, giving a single sharp peak. Successful electron capture gas chromatography of intact bromazepam at the nanogram level has recently been reported by De Silva et al.[74].

E. Oxazolobenzodiazepines

The 5,4-oxazolobenzodiazepine derivatives oxazolam and cloxazolam (Fig. 11) have undergone clinical studies. Their metabolism in the rat has been studied by TLC of the ^{14}C-labelled material in conjunction with IR, UV, NMR and mass spectroscopic data[75,76]. Oxazolam metabolites included desmethyldiazepam, oxazepam and several benzophenones.

In one of the few papers on HPLC of the benzodiazepines to be published so far, Weber[77] described the analysis of the related compound ketazolam. This compound cannot be analyzed using GLC as it is immediately pyrolyzed to give diazepam. This is unacceptable as diazepam is used in the synthesis of ketazolam, and is also a metabolic product. The two compounds were separated on a 1-m Corasil II column
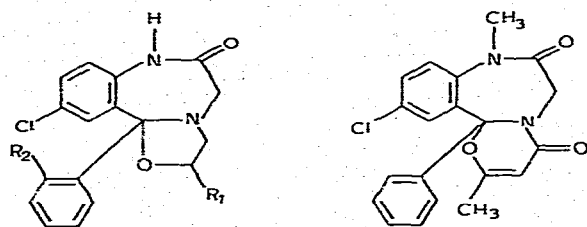
Oxazolam $R_1 = CH_3$, $R_2 = H$ Ketazolam
Cloxazolam $R_1 =$ H , $R_2 = Cl$

Fig. 11. Formulae of oxazolobenzodiazepines.

(Fig. 12) using a mixture of tetrahydrofuran and diisopropyl ether (15:85) as eluent. Detection was by UV absorbance at 254 nm, with a full-scale reading equivalent to 0.02 A. Sensitivity limits (amounts injected on column) were 5 ng for diazepam and 30 ng for ketazolam. Repeated sampling of the test solutions was used to check on the rate of conversion of ketazolam into diazepam in the tetrahydrofuran–diisopropyl
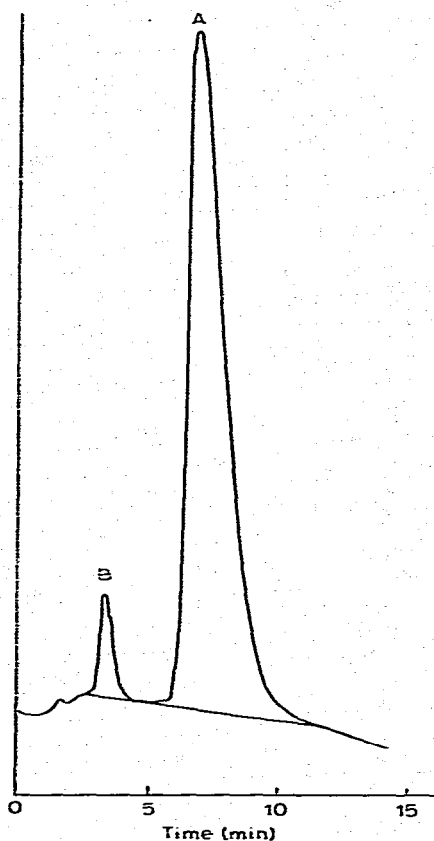

Fig. 12. HPLC separation of ketazolam and diazepam. (Reproduced from ref. 77 with permission of the publishers.)

ether solvent. Extension of the method to oxazepam and nitrazepam gave limits of 15 and 40 ng, respectively. These sensitivities represented approximately a 1000-fold improvement from the levels chromatographed by Scott and Bommer[23] and probably give a good indication of the results to be expected using current commercially available UV detectors.

F. Prazepam

Prazepam (Fig. 13) has been used in clinical trials, although it is not yet marketed. Its metabolites include a number of other benzodiazepines already considered. Prazepam metabolites in dog, rat and mouse have been investigated by Di Carlo and co-workers[78–80] using the 2-^{14}C-labelled compound and TLC. Spots were detected by use of a radiochromatogram scanner, Bratton–Marshall reaction with benzophenone hydrolysis products, or reaction with iodine. Quantitative results were obtained by scintillometry. Prazepam metabolism in man was studied by similar methods[81]. Co-chromatography with known standards was used with a radio-chromatogram scanner.
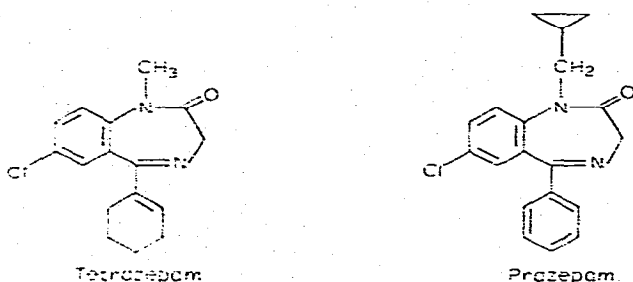

Fig. 13. Tetrazepam and prazepam.

Gas chromatography of prazepam and its metabolites following therapeutic dosage has not yet been reported. However, prazepam itself has extraction and chromatographic characteristics very similar to those of diazepam[29], and electron capture gas chromatography of biological extracts containing the drug and its metabolites using the type of approach already described for diazepam and medazepam should present little difficulty.

G. Lorazepam

Lorazepam is a derivative of oxazepam, differing in having an additional chlorine atom substituted at the 2'-position in the 5-phenyl ring (Fig. 14). It is a more potent drug than oxazepam and consequently both doses and body fluid levels are lower. Following therapeutic dosage, urinary metabolites of the drug were studied by Schillings et al.[82] using TLC methods in conjunction with mass spectroscopy and infrared spectroscopy. Similar methods to those used for oxazepam were employed, incorporating two-dimensional chromatography on silica gel plates, and similar detection techniques. The major urinary metabolite was again the glucuronide, other
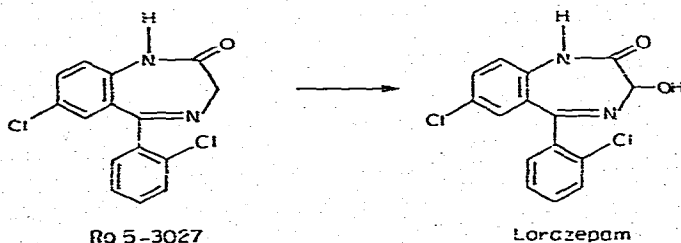
Fig. 14. Ro 5-3027 and lorazepam.

compounds accounting for less than 1 % of the total excreted. Schuetz and Schuetz[53] have described a rapid detection method for the drug in urine by acid hydrolysis, ether extraction of the benzophenone (ADCB), and TLC with the spots being made visible with Bratton–Marshall reagent.

Knowles et al.[54] developed an electron capture gas chromatographic method similar to that for oxazepam. Serum buffered at pH 7 was ether extracted, and the benzodiazepine then back-extracted into 12 N sulphuric acid and hydrolyzed at 100°. The conjugated drug was determined after β-glucuronidase incubation of the extracted aqueous layer. These workers used a 10 ft. × 2 mm I.D. stainless-steel column packed with 3 % OV-17 on Chromosorb W, 100–120 mesh, with a column temperature of 280°. The method was sufficiently sensitive to measure serum and urine concentrations of lorazepam even after 2-mg doses, with a lower level of 0.01 μg/ml.

Marcucci et al.[55] have chromatographed lorazepam intact on 3 % OV-17–Gas-Chrom Q (100–120 mesh) with a sensitivity limit of 1 ng/ml of blood. A 78 % extraction recovery was reported, and BACB was added as internal standard prior to chromatography. Gas chromatography of intact lorazepam has also been discussed by De Silva et al.[56] in connection with the benzodiazepine Ro 5-3027 ("2'-chloro-diazepam"), which has lorazepam as its main metabolite. In this system, hydrolysis to the benzophenone is undesirable because of loss of specificity. Using a method similar to that for medazepam and diazepam[6], it was found that the apparent recovery of lorazepam from blood was only 40 ± 6.0%, which was attributed to adsorption phenomena on column, especially with older columns. Reproducible trimethyl-silyl derivatives could not be prepared. It was also noted that sensitivity to the electron capture detector (ECD) was enhanced on addition of the compounds to blood, compared with the response obtained from standard solutions. This suggested the formation of adsorption complexes or chemical derivatives in the presence of impurities. Similar, but smaller, enhancement could be obtained from addition to 6 N hydrochloric acid. These authors also consider the possibility that on-column con-version to the quinazoline carboxaldehyde may not be quantitative in the presence of impurities.

H. 7-Nitrobenzodiazepines

Several compounds which have been used clinically have higher potencies than that of diazepam, and are therefore often given in lower doses and produce lower blood and tissue levels. These include the compounds containing a 7-nitro group and/or an

additional halogen substituent in the 2'-position. The low concentrations obviously place increased demands on the chromatographic methods, both from the point of view of absolute sensitivity and exclusion of interfering substances. Adsorption, oxidation and photodecomposition become more significant processes at these low levels. Because of these difficulties, it is evident that methods evolved for analysis of the drug in quality control or in overdose situations may not be suitable for clinical work or for pharmacokinetics.

Metabolic pathways for the 7-nitrobenzodiazepines are indicated in Fig. 15. In addition to the routes shown, N-demethylation has also to be considered in the case of flunitrazepam and nimetazepam. The major blood metabolites of nitrazepam and clonazepam have low pharmacological activity[57], and it may often be appropriate in clinical pharmacology to analyse only the parent drug.

The gas chromatography of 7-nitrobenzodiazepines is summarized in Table 4.

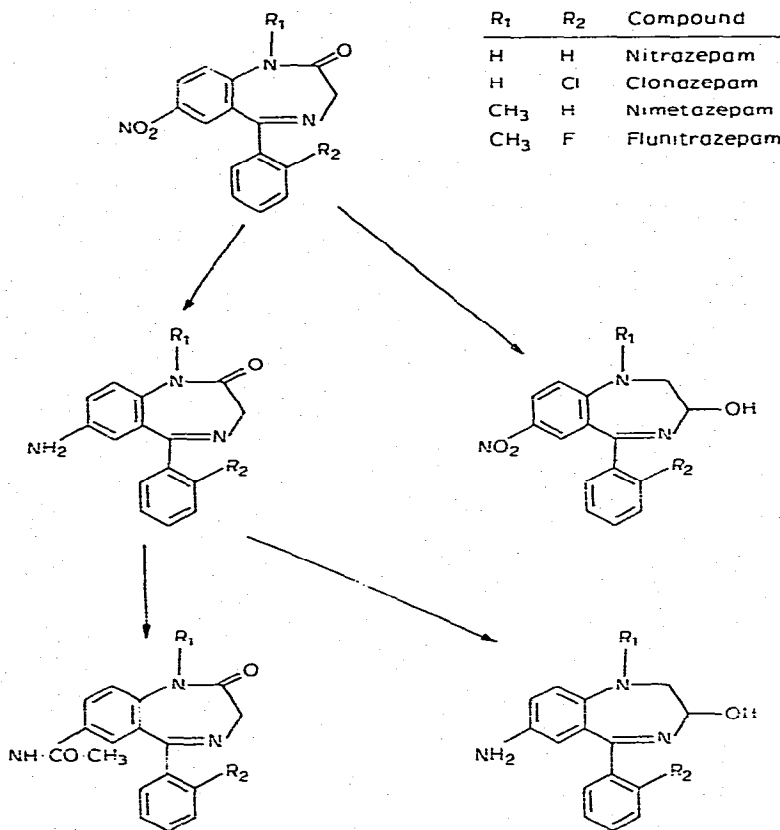| R_1	R_2	Compound
H	H	Nitrazepam
H	Cl	Clonazepam
CH_3	H	Nimetazepam
CH_3	F	Flunitrazepam

Fig. 15. Metabolic pathways for the 7-nitrobenzodiazepines.

(a) Nitrazepam

Nitrazepam is widely used as a sleep inducer and anticonvulsant. As with other benzodiazepines, the metabolism of nitrazepam was studied using TLC sepa-

TABLE 4

GAS CHROMATOGRAPHY OF THE 7-NITROBENZO[D]IAZEPINES

Benzodiazepine	Workers	Column	Detector	Internal standard	Extraction and work-up	Comments
Nitrazepam	Laffargue et al.[100] (1970)	2-m, 3% OV-17 on Gas-Chrom Q, 100–120 mesh $T = 250°$	FID	—	—	Chromatography of intact nitrazepam and two metabolites, and hydrolysis products. Microgram amounts injected on column; poor peak shapes
	Viala et al.[99] (1971)	1.20-m glass, 10% SE-52 on Chromosorb W, $T = 210–270°$ (programme)	FID	—	Diethyl ether or 2:1 dichloromethane–ethyl acetate. Acid hydrolysis to benzophenones	Method for toxicological analysis
	Beharrell et al.[98] (1972)	1.8-m × 4-mm I.D. glass, 3% OV-17 on Gas-Chrom Q, 60–80 mesh, $T = 235°$	[63]Ni ECD	Clonazepam (ANCB)	Diethyl ether, acid clean-up and hydrolysis to benzophenones	Suitable for plasma levels after therapeutic dose. Metabolites not detected
	Ehrsson and Tilly[96] (1973)	0.9-m × 2-mm I.D. glass, 5% OV-17 on Gas-Chrom Q, 80–100 mesh, $T = 250°$	[63]Ni ECD	Griseofulvin	Benzene, then methylation	Suitable for plasma levels after therapeutic dose. Metabolites not detected
Clonazepam	Naestoft et al.[102] (1973)	3-ft. × 4-mm I.D. glass, 1% OV-17 on Celite CQ, 100–120 mesh	[63]Ni ECD	Desmethyl-diazepam	Toluene–isoamyl alcohol, acid clean-up	Plasma levels after therapeutic dose. Possible interference from metabolite. Intact drug chromatographed
	Naestoft et al.[103] (1974)	4-ft. × 4-mm I.D. glass, 3% OV-17 on 60–80 mesh Gas-Chrom Q, $T = 230°$	[63]Ni ECD	(1) Desmethyl-flunitrazepam (2) Acetylated Ro 10-3384	Ethyl acetate, acid clean-up and differential extraction	Suitable for chromatography of drug and two major metabolites after therapeutic dosage
	De Silva and Puglisi[104] (1974)	4-ft. × 4-mm I.D. glass, 3% OV-17 on 60–80 mesh Gas-Chrom Q, $T = 230°$	[63]Ni ECD	Flunitrazepam (MANFB)	Diethyl ether extraction, acid clean-up and hydrolysis to benzophenones	Used for blood level determinations following therapeutic dosage. Metabolites not detected
Flunitrazepam	De Silva and Puglisi[104] (1974)	3-ft. × 4-mm I.D. glass, 3% OV-225 on 60–80 mesh Gas-Chrom Q, $T = 230°$	[63]Ni ECD	Clonazepam (ANCB)	As above	Blood level determinations of drugs and desmethyl metabolite

ration of the ^{14}C-labelled compound. The spots were rendered visible by auto-radiography and the eluted radioactive material was measured by scintillometry. In 1965, Rieder[88] published TLC data on nitrazepam and its two major blood metab-olites. The spots were made visible with either Folin's reagent (grey-blue spot) or Bratton–Marshall reagent (blue-red), which gave more sensitive detection. Pribilla[89] also used TLC to study the excretion of nitrazepam in humans.

Oelschlager and co-workers[90,92,93] separated nitrazepam and its metabolites on magnesium silicate plates and determined the compounds by d.c. polarography. Areas of adsorbent containing the compounds were scraped off and shaken with dimethylformamide (to reduce adsorption) before analysis.

Scott and Bommer[28] and Weber[77] chromatographed nitrazepam with HPLC systems using UV detection.

Gas chromatography of nitrazepam was reported by Matsuda[91], who obtained usable chromatograms for the hydrolysis product 2-amino-5-nitrobenzophenone (ANB), with flame ionization detection. Nitrazepam gave a broad peak with pro-nounced tailing. High concentrations were chromatographed, and the method was not suitable for the determination of the drug following therapeutic doses. Marcucci et al.[13] included nitrazepam in their work on diazepam chromatography, but once again high levels were chromatographed and no work was carried out on detecting the drug in biological fluids. Hydrolysis to ANB was also described by Viala et al.[69], who followed the work of Rieder[88] in developing toxicological analytical methods for the drug and its metabolites using TLC with a Bratton–Marshall reaction. Gas chromatography of ANB was carried out on 10% SE-52 on Chromosorb W using flame ionization detection.

In 1972, Beharrell et al.[95] reported a method for determining nitrazepam in biological samples using an OV-17 stationary phase and electron capture detection, which was capable of quantitation of the drug following therapeutic doses. Clonaze-pam was used as an internal standard. Reproducible results could not be obtained with the intact molecules below about 1.0 μg/ml, and hydrolysis with hydrochloric acid to the benzophenones (ANB and ANCB) was therefore used. The results are shown in Fig. 16. All glassware was silanized to minimise adsorption processes. The method was not suitable for the amino and acetamido metabolites, which are weakly electron capturing and hydrolyze to a benzophenone (DAB), which also has a low affinity for electrons. Ehrsson and Tilly[96] reported an electron capture GC method for nitrazepam which eliminated the need for acid hydrolysis. This was made possible by methylation of the N_1 position using iodomethane–tetrabutylammonium hydrogen sulphate after benzene extraction of the drug. Glassware was silanized before use. The major metabolites did not interfere in the chromatography, being well separated from methylnitrazepam and the internal standard, and also giving a much lower ECD response. The method was suitable for quantitation of nitrazepam in the range 5–100 ng/ml (Fig. 17).

It seems probable that the GLC of nitrazepam will develop in similar fashion to that of diazepam. With improved column technology, it should be possible to chromatograph the compound intact even at the low levels (1–20 ng/ml) expected after therapeutic doses, and thereby eliminate the necessity for hydrolysis or methyl-ation. Increased ECD sensitivity to the 7-amino metabolites can be achieved by reaction with pentafluoropropionic anhydride to give the fluoroacyl derivative[97]. In
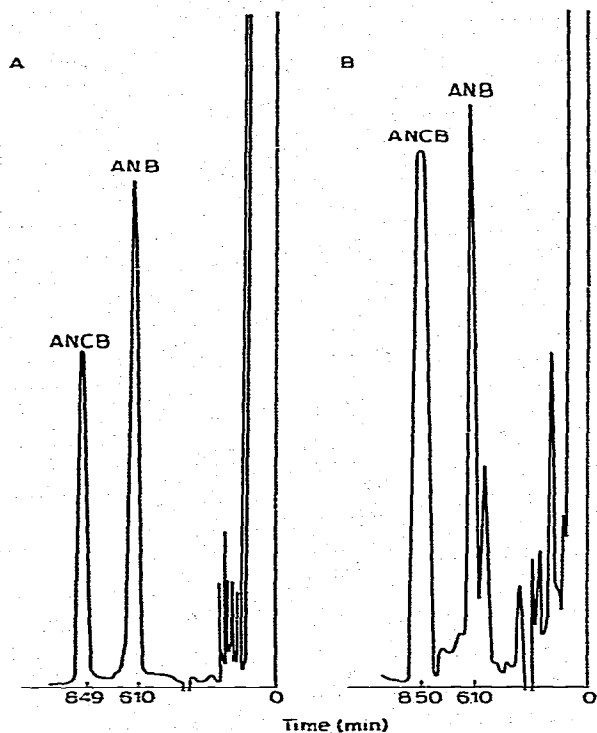
Fig. 16. Chromatograms of ANB–ANCB mixtures. (A) standard solution. ANB concentration 0.04 µg/ml: (B) plasma sample. ANB concentration 0.025 µg/ml. 3 % OV-17 stationary phase. (Reproduced from ref. 95 with permission of the publishers.)
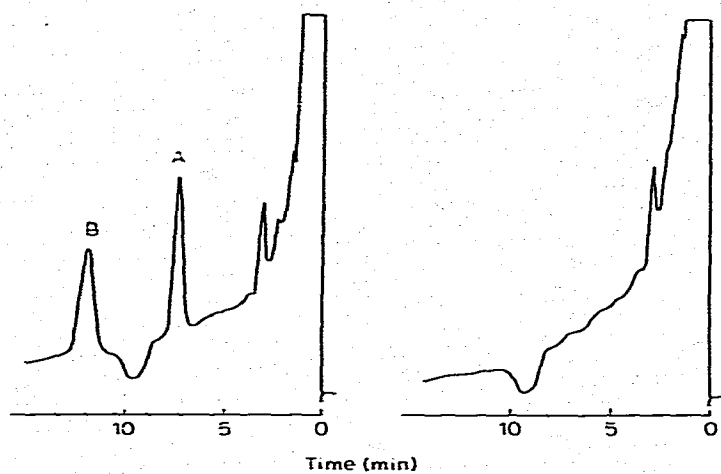

Fig. 17. (a) Gas chromatogram from human plasma containing 40 ng/ml of nitrazepam. A = methylnitrazepam, B = griseofulvin: (b) chromatogram from human blank plasma (no addition of internal standard). 5 % OV-17 stationary phase. Electrometer setting: 4×10^{-10}. (Reproduced from ref. 96 with permission of the publishers.)

most of the work carried out so far on the determination of nitrazepam metabolites in body fluids, the fluorimetric method described by Rieder[98] has been used.

It is of interest to compare the different electron capturing ability of nitrazepam and its metabolites with the situation for medazepam mentioned previously. In the case of nitrazepam, removal of the electronegative substituent at position 7 (by reduction) greatly reduces the ECD response, although the diazepin-2-one structure is unchanged. Medazepam has a relatively weak electron-capturing ability, despite the presence of a halogen atom at C_7. Introduction of the carbonyl group (giving diazepam) greatly increases the response.

(b) Clonazepam

Clonazepam has been used clinically as an anticonvulsant. As with nitrazepam, body tissue and fluid levels are very low, making demands on the analytical method. TLC methods were used to elucidate the metabolism of the compound[99,100].

A method for the intact drug in human serum following administration of 2- and 4-mg doses was described by Næstoft et al.[101,102]. These workers extracted the drug from plasma with toluene containing isoamyl alcohol, and following a clean-up procedure with hydrochloric acid and heptane, used electron capture gas chromatography with desmethyldiazepam as an internal standard (Fig. 18). Interference
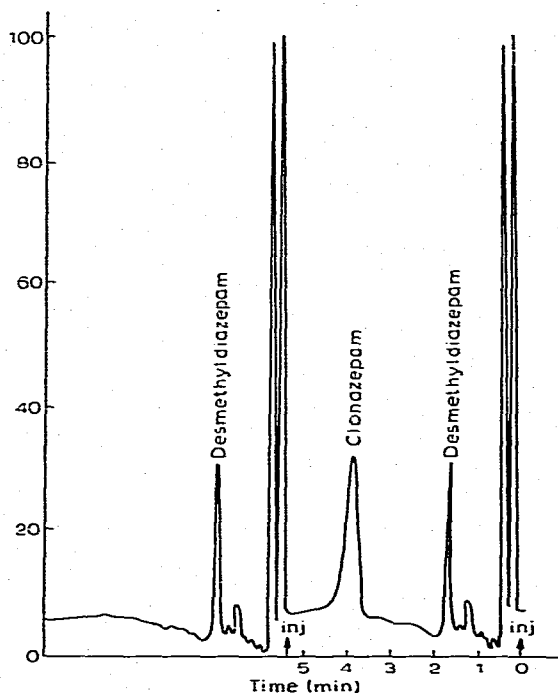

Fig. 18. Chromatograms of an extract from a plasma sample from a patient not receiving clonazepam, blind with added internal standard (left), and an extract from a plasma sample from one patient receiving clonazepam (right). 1 % OV-17 stationary phase. (Reproduced from ref. 102 with permission of the publishers.)

by the 7-amino metabolite was considered a possibility. Subsequently, Næstoft and Larsen[102] improved the method to make possible the separate quantitation of clonazepam and both major metabolites. In the modified assay, clonazepam is first separated from its metabolites by differential extraction. It is then chromatographed on OV-17 with desmethylflunitrazepam as internal standard. The two metabolites are chromatographed separately on the same column with 7-acetamido-2′-chloro-1-methyl-1,4-benzodiazepin-2-one as internal standard. Detection limits were 3–5 ng/ml.

In contrast to this work, De Silva *et al.*[104] found that electron capture detection of intact clonazepam gave unsatisfactory results, broad and poorly defined peaks being obtained on both OV-1 and OV-17 phases. Hydrolysis to ANCB gave a well defined peak, and was capable of being used to assay clonazepam at the nanogram level. Possible interference due to the presence of the 3-hydroxy metabolite (which also hydrolyzes to ANCB) was eliminated because of the different partition characteristics of this compound during the extraction procedure. Flunitrazepam (Ro 5-4200) was used as an internal standard, this compound hydrolyzing to the benzophenone MANFB. Using an OV-17 stationary phase, the detection limit for blood samples was between 0.5 and 1.0 ng/ml, using a 2-ml sample. For optimum electron capture detection, it is important that trace amounts of water are not introduced on injection of the sample. Extracted residues were therefore vacuum dried and dissolved in acetone–hexane which had been dried over anhydrous sodium sulphate. Quantitative extraction was achieved with ether from borate buffered plasma. The method was not suitable for the 7-amino metabolite of clonazepam, as hydrolysis gave an unexpected product with a very poor electron capture response, more than 200 ng being required to produce a measurable peak.

This method for clonazepam was associated with a similar one for flunitrazepam (Ro 5-4200) and its desmethyl metabolite, clonazepam in this second assay being used as internal standard. Apparent recoveries of Ro 5-4200 from blood were greater than 100%, suggesting that complex formation with blood-extracted impurities occurred. Flunitrazepam and its desmethyl metabolite are eluted intact on a GLC column with good peak shape, but a benzophenone method is required as both are unstable in acid, which is used in the essential clean-up procedure. In the flunitrazepam assay, OV-225 was used as stationary phase rather than OV-17, giving a better separation of the three benzophenones MANFB, ANFB and ANCB.

The hydrolysis medium used in both assays was a mixture of 4 N hydrochloric acid and 4 N sulphuric acid (1:1), as hydrochloric acid alone sometimes resulted in chlorination of ANCB. This was especially true with old hydrochloric acid, and it was suggested that an increase in free oxygen concentration gave rise to free or activated chlorine in solution. Trace metal-catalyzed chlorination was another possibility. In the case of MANFB and ANFB, it is essential to carry out the neutralization step following the hydrolysis carefully at low temperature (ice cooling) as the 2′-fluorobenzophenones are readily converted into the acridones on warming in a basic medium. These observations may have relevance to other benzodiazepine assays which involve hydrolysis to the benzophenones.

In addition to the GLC assay, De Silva and Puglisi[101] analyzed major urinary metabolites of clonazepam and flunitrazepam by TLC linked to pulse polarography. A one-dimensional separation on silica gel was carried out and the compounds were eluted with two 5-ml portions of methanol and dissolved in 0.1 N hydrochloric acid

for polarographic analysis. Using the wave due to reduction of the azomethine moiety (-0.6 V *versus* SCE), the detection limit was 0.5–0.75 μg with a 5-ml sample. This is considered to be suitable for the analysis of urinary metabolites following chronic administration of the drugs.

I. Flurazepam

Flurazepam differs from other benzodiazepines currently marketed in having a large substituent (diethylaminoethyl) at N_1 (Fig. 19). The most important metabolic processes involve degradation of this side-chain, 3-hydroxylation playing a relatively minor role[105]. The major blood metabolites are the hydroxyethyl and desalkyl compounds, flurazepam itself being rapidly eliminated from the circulation. The levels following a therapeutic dose are very low (1–15 ng/ml), and gas chromatographic techniques have been only partially successful in the analysis of these compounds. De Silva and Puglisi[106] developed an electron capture gas chromatographic method based on the benzophenones obtained by acid hydrolysis. Although this method was useful for toxicological work, it was not suitable for use with humans who had
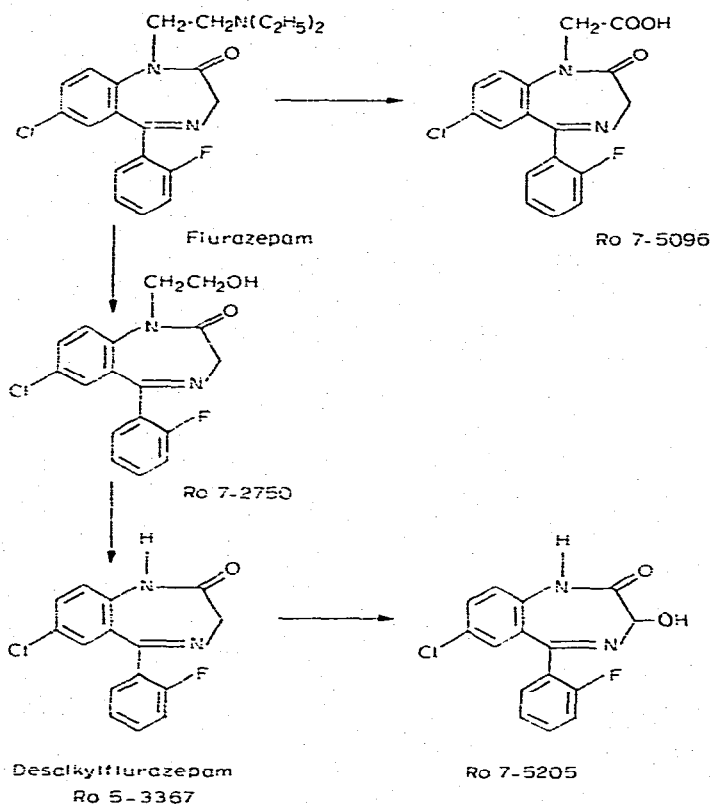

Fig. 19. Metabolic pathways of flurazepam.

received therapeutic doses of flurazepam, as the limit of sensitivity was of the order of 0.02 µg/ml of plasma.

A more sensitive assay was therefore developed in which flurazepam and its metabolites were determined fluorimetrically following TLC separation[107]. The compounds were selectively extracted into ether, back-extracted into 4 N hydrochloric acid, and hydrolyzed to the benzophenones. The hydrolysate was made alkaline, and extracted with ether. The residue obtained after evaporation was dissolved in dimethylformamide, potassium carbonate added, and the mixture heated at 110° for 2 h to convert the benzophenones to the highly fluorescent 9-acridones. Following re-extraction, these were separated by TLC on silica gel plates using chloroform–acetone (85:15) as solvent. The areas on the TLC plate corresponding to the acridones was scraped off, the acridones eluted with methanol–0.1 N hydrochloric acid (80:20), and their fluorescence measured with a spectrofluorimeter.

In the analysis of urinary metabolites, it is sometimes possible to determine the major metabolites by TLC of the benzophenones followed by spectrophotometry, but the levels of the compounds are often too low, and it is then necessary to resort to the more sensitive fluorimetric assay.

Schwartz and co-workers[105,108] used combined TLC and mass spectrometry to identify urinary metabolites of flurazepam. Separation was carried out on fluorescent silica gel and a solvent consisting of ethyl acetate–ethanol–12 N ammonia (90:10:0.3) for the first dimension. A variety of other solvents was used in the isolation of each metabolite. Detection was achieved by spraying with modified Dragendorff reagent. The metabolism of flurazepam in man was followed by similar chromatography of ^{14}C-labelled material. The spots were scraped off the plate after development and counted by scintillometry.

The fluorimetric assay does not distinguish between N-desalkylflurazepam and the desalkyl-3-hydroxy metabolite, as both give rise to the same acridone. However, Kaplan et al.[109] were able to show that the 3-hydroxy compound was not present in the blood of subjects who had received 30 mg flurazepam orally for 2 weeks. An electron capture GC procedure was used that completely resolved the two compounds, and this confirmed that the N-desalkyl compound was the major blood metabolite.

3. CHROMATOGRAPHIC ANALYSIS OF THE BENZODIAZEPINES IN THE BULK DRUGS AND IN FORMULATIONS

Many chromatographic methods have been described for the analysis of the drugs, their decomposition products and their intermediates for quality control purposes[110–130].

Beckstead and Smith[117] described in considerable detail a TLC scheme for the detection of impurities in benzodiazepines. These authors also included a brief review of the earlier literature on the TLC of chlordiazepoxide, diazepam, nitrazepam and oxazepam. TLC methods for these four drugs and their impurities were then described. A number of solvent systems and spray reagents were investigated to separate the benzodiazepines from intermediates in their synthesis and their hydrolysis or decomposition products. The variation in polarity between the compounds ruled out a single TLC system, and a series of systems was developed for quality control handling of each benzodiazepine (Table 3). Silica gel containing a fluorescent indicator was found

to be the most useful adsorbent, and fluorescence quenching was a good general method of detection, detection limits being better than 1 μg. Of the spray reagents, chlorine–o-toluidine and modified Dragendorff–cerium(IV) sulphate were the most sensitive and generally applicable. Bratton–Marshall reagent gave high sensitivity for compounds with a primary amino group. Iodoplatinate reagent was considered unsatisfactory because of the wide variation in sensitivity with related compounds. The scheme was used to analyse a number of commercially available benzodiazepine formulations and bulk drug samples.

Arizan et al.[119] used TLC and polarography to study various stages in the synthesis of diazepam. Dragendorff reagent was used to render the spots visible, polarography being used for quantitation. Bich et al.[120] used a TLC–spectrophotometer system to measure individual active agents in drug formulations, including chlordiazepoxide, eliminating the need to elute the compounds from the plate. The sensitivity was in the microgram range. Mayer et al.[121] used TLC techniques to study the hydrolysis and decomposition of diazepam and nitrazepam during storage and showed that the rate of decomposition/hydrolysis depended on the moisture content and the presence of auxiliary ingredients with a large surface area. TLC methods for chlordiazepoxide and diazepam have been summarized by MacDonald et al.[126].

Haefelfinger[129] used the reagent 2,5-dimethoxytetrahydrofuran–p-dimethyl-aminobenzaldehyde to locate primary amines on TLC plates. It was noted that both nitrazepam and diazepam gave positive results, unlike medazepam or chlordiazepoxide. The anomaly was explained by suggesting that with the two reacting compounds, the diazepine ring opened, giving a primary amino group. Analytical methods for determination of stability of drug parenterals have been discussed by Johnson and Venturella[122], who included the GLC and TLC analysis of diazepam.

Fricke[125] has described a semi-automated GLC procedure for a variety of drugs, including chlordiazepoxide and diazepam, using Dexsil 300 as the stationary phase. Lafargue et al.[130] described a GLC study of a number of benzodiazepines and some of their metabolites and hydrolysis products. Sample sizes of 2 or 4 μg were injected on a column of OV-17 on 100–120 mesh Gas-Chrom Q using flame ionization detection. The compounds studied included chlorazepate, which was impossible to chromatograph, and tetrazepam, which was eluted between oxazepam and diazepam. The nitrazepam metabolites Ro 5-3072 and Ro 5-3308 were eluted intact but with poor peak shape and pronounced tailing. Desmethylchlordiazepoxide gave three peaks, indicating thermal instability. Retention data were included for all compounds studied.

4. CHROMATOGRAPHIC ANALYSIS OF THE BENZODIAZEPINES IN CLINICAL TOXI-
   COLOGY

A great number of general screening procedures for drugs in urine and blood, ncluding one or more benzodiazepines, have been published[131–162]. In many cases, metabolites were not taken into consideration in these methods, and the benzophenone hydrolysis products were chromatographed rather than the intact drugs. A comprehensive bibliography of these screening methods has not been included in this review, but the literature cited here is thought to cover the most important work in this field. There are also a number of publications which deal with the benzodiazepines

alone[69,73,91,163-175], and which contain methods to distinguish between the different compounds and their metabolites. The various TLC systems used in benzodiazepine analysis have tended to use rather similar methods of development and detection, and the paper by Beckstead and Smith[117] referred to previously provides a useful summary of the earlier work.

The cheapest and most widely used screening procedures have used TLC systems, usually in conjunction with spray reagents. Fluorescence quenching has also been popular. In addition, many of the benzodiazepines rearrange to highly fluorescent products on treatment with concentrated acids, giving rise to very sensitive detection methods (Table 5).

TABLE 5

FLUORESCENCE DATA FOR BENZODIAZEPINES
From Lafargue et al.[173]

Compound	Acid	$\lambda_{max.}$ excitation (nm)	$\lambda_{max.}$ emission (nm)
Chlordiazepoxide	H_2SO_4	310	530
Diazepam	H_2SO_4	295	490
Chlorazepate	H_2SO_4	388	508
Medazepam	H_2SO_4	345	485
Oxazepam	H_3PO_4	360	475
Nitrazepam	$HClO_4$	300	465
Tetrazepam	H_3PO_4	398	492

Bellemonte[167] used UV and IR spectrophotometry in conjunction with TLC to determine diazepam and its metabolites in urine, following hydrolysis to the benzophenones. Oelschlager mentioned the TLC separation of chlordiazepoxide, diazepam and nitrazepam and their metabolites in an early review of the compounds[112], and reported d.c. polarography as being suitable for quantitation. Zingales[137] used an ether extraction followed by TLC using five chromatographic systems and several colour reactions to identify a number of psychotropic drugs, including diazepam and chlordiazepoxide. Weist and Schmid[142] described a rapid TLC method for benzodiazepines and other drugs on micro-plates, which was used for the detection of the compounds in cases of poisoning.

De Silva and D'Arconte[169] mentioned the use of TLC linked to fluorimetry in the forensic analysis of chlordiazepoxide, and on-plate spectrofluorimetry was used by Lauffer and Schmid[141] in their separation scheme for 60 drugs, including chlordiazepoxide. Alha and Lukari[170] used diazotisation and coupling of 7-amino-nitrazepam and ACB in the TLC of forensic samples. For quantitative work, the spots were eluted and measured spectrophotometrically. Sawada and Shinohara[172] used a TLC system to identify nitrazepam and its metabolites in *post mortem* samples. Using a series of solvents and spray reagents, the method was also applied to chlordiazepoxide, oxazepam and diazepam. Lafargue et al.[173] gave details of a rapid method for benzodiazepines in toxicological cases, using fluorescent alumina plates to separate the hydrolysis products. Metland et al.[157] used XAD-2 ion-exchange resin to remove the drugs from urine before elution and TLC with a number of spray reagents. Schuetz et al.[175] have described the two-dimensional TLC of five benzodiazepines, the compounds being converted into the benzophenones on plate by

spraying with hydrochloric acid, and then being reacted with Bratton–Marshall reagent. Berry and Grove[161] identified benzodiazepines in urine by heating the samples with 1 N hydrochloric acid in an autoclave at 15 p.s.i. for 15 min, and then extracting with light petroleum, chromatographing the benzophenones on silica gel, and coupling with N-naphthylethylenediamine to give an azo dye. The method is sensitive, being capable of detecting urine levels after therapeutic dosage, but is limited in specificity as oxazepam, chlordiazepoxide and desmethyldiazepam give the same hydrolysis product (ACB). Medazepam and diazepam are not detectable by this method. Nitrazepam hydrolyzes to the benzophenone ANB, which is separable from ACB. In addition, the 7-acetamido metabolite of nitrazepam gives a blue fluorescence under UV light after chromatography with methanol–12 N ammonia (100:1.5).

Systems based on GLC using flame ionization detection, or GLC in combination with TLC, have also been widely used. Vignoli and Cano[161] chromatographed ACB, MACB and ANB on an SE-52 stationary phase using FID, reporting detection limits of 60 ng for diazepam and nitrazepam and 90 ng for chlordiazepoxide. Finkle et al.[152] included chlordiazepoxide, diazepam, medazepam, oxazepam, flurazepam and nitrazepam in their screening procedure based on direct solvent extraction and a GLC system utilizing four columns and three stationary phases. A sensitivity limit of 2 μg/ml was reported. Flurazepam was chromatographed at 250°, and the other three compounds at 200° on an SE-30 stationary phase. The primary purpose of this work was to provide retention data for a large number of drugs. Proelss and Lohmann[151] included chlordiazepoxide, diazepam and oxazepam in a screening method for 40 sedatives and tranquillisers. Benzodiazepines were ether extracted from buffered serum at pH 8. A number of stationary phases were evaluated, and the best results were obtained with 3% OV-17, this being the only phase which resolved all clinically important phenothiazines, dibenzazepines and benzodiazepines. Phenothiazine was used as an internal standard.

Viala et al.[69] described a combined GLC and TLC procedure for the toxicological analysis of benzodiazepines in blood and urine. Account was taken of the major metabolites, and the compounds chlordiazepoxide, diazepam, medazepam, nitrazepam, chlorazepate and oxazepam were included. Most of the work made use of hydrolysis to the benzophenones in order to obtain increased sensitivity by GLC. As a number of benzodiazepines give ACB as a hydrolysis product, GLC of the intact compounds was also used for identification purposes. Flame ionization detection was used in most cases, although electron capture detection was mentioned for the analysis of ACB, MACB, medazepam and desmethyldiazepam. A TLC system on silica gel plates was also described. Interferences by overlapping benzodiazepine spots could be eliminated using different detection methods. Interference by other drugs was not considered.

Sine et al.[155] used GLC with flame ionization detection following chloroform extraction of serum buffered at pH 7.4. A caffeine internal standard was used, and the column contained 3.8% SE-30 on Chrom W. Gardner-Thorpe et al. published TLC[149] and GLC[156] systems for determining anticonvulsants in blood and included chlordiazepoxide, diazepam, nitrazepam and oxazepam. TLC on silica gel gave detection levels of less than 1 μg for the benzodiazepines when viewed under UV light. In the GLC work, several columns were evaluated and retention data reported. No

suitable system for nitrazepam was obtained. The other benzodiazepines were separated on 3% SE-30 at 250° and detected using flame ionization.

Law *et al.*[154] described a GC–MS system with computer storage for low-resolution mass spectra of 58 drugs including chlordiazepoxide and diazepam. The method was based on comparison between *m/e* values of the five strongest peaks in the mass spectra and both drugs were detected in serum from subjects following drug overdose. Finkle and Taylor[153] have also published details of a GC–MS reference system for drug identification which included chlordiazepoxide. diazepam and medazepam. Data were obtained by chromatography on 2.5% SE-30 followed by detection with a quadrupole mass spectrometer. Mass spectral data were numerically coded and compared with stored reference data for final identification. Simple chloroform extractions were used. and the mass spectra references were obtained by injection of 50–100 ng of drug on the column. Unknown peaks were matched by a.m.u. value of the mass spectral base peak and by the most intense peak in every 14 a.m.u. from 43 to 463 *m/e*.

Greaves[73] has reported the quantitative determination of medazepam. diazepam and nitrazepam in whole blood by flame ionization GLC. The method was suitable for toxicological analysis of the compounds following overdosage or chronic therapeutic administration in the case of medazepam and diazepam. OV-1 and OV-17 stationary phases were used. Blood samples were ether extracted on a vortex mixer and. after acid clean-up. the combined extracts were passed through anhydrous sodium sulphate before evaporation. Nitrazepam was reacted with BSTFA to form a TMS derivative which was eluted on OV-17 with the same retention time as diazepam. It was shown that a number of other commonly used drugs did not interfere in the assay.

5. FUTURE TRENDS

It is very probable that a number of other benzodiazepines will before long be available for clinical use.

A number of the new compounds that can be expected to be marketed in the future will be administered in low doses because of increased potency compared with earlier drugs of this type. Flunitrazepam has already been referred to. and the 6-phenyl-4H-5-triazolo[4.3-*a*] compounds have also shown high activity and low toxicity[176]. Analytical methods for these compounds will need to be highly sensitive and selective. As with benzodiazepines that are already available clinically, a number of metabolites will be pharmacologically active, and methods for their determination will need to be developed. Consideration of the chemistry of the 1.4-diazepine ring system and of interferences to be expected from other compounds will be important in the development of chromatographic and other analytical methods.

The possibility of complex formation with biological material, as suggested by De Silva and co-workers[86,104]. needs fuller investigation. Further work might also be done in developing more selective procedures for extraction of the drugs and metabolites from body fluids. Use of a structurally similar benzodiazepine as an internal standard for both extraction and chromatography is considered highly desirable. especially in the analysis of the drugs at concentrations less than 20 ng/ml.

Prescription of any psychoactive drug implies eventual misuse and overdosage

by a proportion of the population. Overdosage of benzodiazepines is at present very common[177], and the introduction of further compounds of this type will increase the work of the clinical toxicologist. Revised TLC screening methods will be needed, and for some of the more potent drugs, very sensitive spray reagents highly desirable. Conversion to fluorescent derivatives such as acridones will continue to find application. Caille et al.[178] have recently characterized the fluophore produced by treatment of ethanolic solutions of oxazepam with phosphonic acid, and shown it to be the trimer of the thermolysis product 6-chloro-2-formyl-4-phenylquinazoline. Some of the benzodiazepines with more complex structures may not be so amenable to gas chromatography as the earlier members of the series, and TLC followed by formation of a suitable fluophore will continue to provide a useful alternative. De Silva et al.[179] have described the TLC–fluorimetric assay of an indolyl-1,4-benzodiazepine, making use of conversion to the fluorescent quinolone after treatment with sulphuric acid.

Gas chromatography is likely to continue to be of major importance for benzodiazepine analysis. Electron capture detection will probably retain its place, especially for the more potent drugs, as the most useful method for monitoring blood and urine levels after therapeutic dosage. Introduction of newer designs of electron capture detector, with a wide linear range and possibly better sensitivity, will make the application of this technique easier. For the analysis of benzodiazepines that give higher body fluid and tissue levels, thermionic and conductivity detectors could become far more widely used, being potentially attractive in terms of the simple extraction and clean-up procedures required. GLC–MS has obvious attractions, especially when used for mass fragmentography. With a favourable fragmentation pattern, good sensitivity and very high selectivity can be achieved, and there is the possibility of being able to use simple extraction procedures without further clean-up, even for very low levels of the drug. This could be particularly advantageous in the case of compounds such as flunitrazepam, which decompose in acid.

In addition to GC–MS applications, use of direct mass spectrometric analysis seems a possibility. Boerner et al.[180] have recently reported the use of this technique in analyzing drugs in body fluids in acutely poisoned patients. A chemical vapour analysis system with a computer-linked quadrupole mass spectrometer was used. Drugs analyzed included benzodiazepines. This method has the advantage over chromatographic techniques in being very rapid. This could be of use in the management of some overdosage cases where identification of the drug of abuse is important. However, the technique does not have the sensitivity of some chromatographic methods, and involves the use of expensive instrumentation which may not be available to the hospital laboratory. This method could also find application in metabolic studies. Direct mass spectrometry of a number of glucuronides, including that of oxazepam, has been reported by Billets et al.[181].

The use of HPLC seems likely to increase, especially with the availability of less expensive apparatus and more sensitive detectors such as fluorimeters. This technique (in its ion-exchange mode) should be particularly suitable for the analysis of urinary metabolites, many of which have so far not been identified. Even with currently available detectors, blood level determinations of a number of benzodiazepines would seem to be feasible.

For the routine determination of marketed benzodiazepines, immunoassay would appear to offer a number of advantages. Peskar and Spector[182] have developed

a radioimmunoassay for diazepam and desmethyldiazepam. A detection limit of about 1 ng was achieved with a linear range of 1–100 ng. A possible disadvantage of such a method is cross-reaction with other benzodiazepines or metabolites. In the case of the antisera used by Peskar and Spector, it was shown that medazepam, desmethylmedazepam, chlordiazepoxide, demoxepam and oxazepam did not bind to the antibody. However, the binding of compounds with a 2-carbonyl function and no 3-hydroxy or 5-N-oxide substituent (e.g., nitrazepam) were not reported.

Polarographic methods should provide a useful alternative to GLC for quantitation of benzodiazepines, especially in overdose situations. The technique has been used by a number of workers. For example. Berry[183] has used polarography in the determination of diazepam and chlordiazepoxide in plasma and urine. and Halvorsen and Jacobsen[184] have measured nitrazepam and metabolites in horse plasma. Limitations of the technique are sensitivity, which is not yet as good as that of the best GLC detectors, and resolution of the drugs and their metabolites. Resolution of electrochemically very similar benzodiazepines generally requires a prior TLC separation[61,94] or a selective extraction technique.

The analysis of benzodiazepines will continue to demand skilled application of modern analytical techniques. Chromatographic methods can be expected to play a major part in the analysis of these drugs.

6. ACKNOWLEDGEMENT

7. SUMMARY

A review is presented of chromatographic methods of analysis for the 1,4-benzodiazepine drugs. Particular emphasis is placed on their determination in biological materials, with discussion on the difficulties involved in this type of work. A short section on chromatographic methods in quality control is included. and the review is concluded by a brief discussion of future developments in the field.

NOTE BY EDITOR

After the submission of this manuscript. a review by J. M. Clifford and W. F. Smyth was published [*Analyst (London)*. 99 (1974) 241] on the determination of 1,4-benzodiazepines and their metabolites in body fluids. The two reviews overlap as far as the chromatographic aspects of the analysis of body fluids is concerned.

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