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TRACE ANALYSIS OF BENZODIAZEPINE DRUGS IN BLOOD USING DEACTIVATED AMBERLITE XAD-7 POROUS POLYMER BEADS AND SILICA CAPILLARY COLUMN GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A general method for the trace analysis of benzodiazepine drugs and their major metabolites at single dose therapeutic levels in 0.2 ml blood samples is described. The method involves solvent extraction of blood with toluene, isolation of the analytes using deactivated Amberlite XAD-7 porous polymer beads, and analysis of the cleaned-up extracts by capillary column gas chromatography with electron-capture detection. The clean-up technique eliminates lipids and other interfering material, enabling routine analysis of blood extracts to be carried out with no significant deterioration in column or detector performance over a period of many months. The use of fused-silica capillary columns coated with SE-52 and the correct choice of chromatographic conditions permits underivatised benzodiazepines of widely differing volatilities and polarites to be analysed. Data for 26 benzodiazepines and metabolites are presented.

INTRODUCTION

The benzodiazepines (Fig. 1, Table I) are an important class of sedative hypnotic drugs and are widely prescribed throughout the world for the treatment of anxiety, epilepsy and insomnia[1,3,19]. The trace analysis of benzodiazepines in blood is important in forensic science since these compounds are frequently encountered in drugs and driving cases and are also used by criminals to incapacitate their victims[20–22].

The detection of single dose therapeutic levels of unknown benzodiazepines and their metabolites in small blood samples is required in a significant proportion of cases encountered in the Metropolitan Police Forensic Science Laboratory[20–22]. Very sensitive and selective methods are necessary for these analyses because the benzodiazepine levels are mostly in the low ng/ml range (Table I)[23].

Radioimmunoassay (RIA) is currently the only technique that is capable of screening small blood samples[24]. However, RIA gives no indication of the identity of the benzodiazepine or metabolite detected and positive results must be confirmed by an alternative method.

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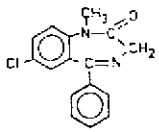
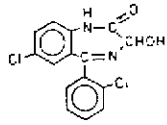


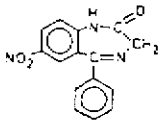
Diazepam

(Valium)



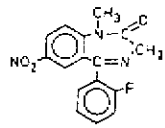
Lorazepam

(Ativan)



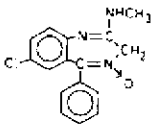
Nitrazepam

(Mogadon)



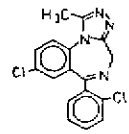
Flunitrazepam

(Rohypnol)



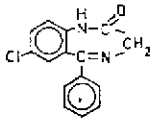
Chlordiazepoxide

(Librium)

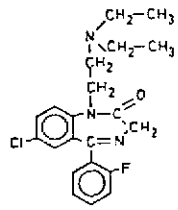


Triazolam

(Halcion)

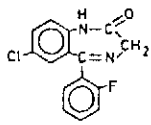


N-desmethyldiazepam



Flurazepam

(Dalmane)



N-desalkylflurazepam

Fig. 1. Important benzodiazepines and metabolites of forensic interest.

High-performance liquid chromatography cannot be used for most benzodiazepines because currently available detectors are insufficiently sensitive and selective[25,26]. The most sensitive and specific routine analytical technique at present available is gas chromatography with electron-capture detection (GC–ECD)[25,27]. In principle, this method is capable of detecting picogram amounts of most benzodiazepines. However, the potential sensitivity of the method has not been achieved due to ad-

sorption and catalytic decomposition of the more polar benzodiazepines on the packed columns that have been used almost exclusively for this analysis[25,28,29]. Many methods have been introduced to overcome the adsorption problem such as priming[29], derivatisation[26], acid hydrolysis of benzodiazepines to benzophenones[26,30], and the use of glass SCOT columns in combination with a solids injector[26]. These methods are useful for pharmacokinetic studies or the rapid identification of individual benzodiazepines taken in overdose quantities, but they lack the necessary sensitivity and resolution for forensic work.

This paper describes a method that overcomes these problems by using deactivated Amberlite XAD-7 porous polymer beads to remove interfering co-extractives, followed by silica capillary column GC–ECD. The Amberlite XAD-7 clean-up improves the selectivity of the ECD and protects the column and detector from contamination, enabling single dose therapeutic levels of a wide range of benzodiazepines and metabolites in 0.2 ml blood samples to be analysed. A similar approach has been used previously to analyse biological extracts for low nanogram levels of underivatised explosives[31].

The method of analysis was developed primarily for use in forensic toxicology, but it is equally applicable to pharmacokinetic studies and therapeutic drug level monitoring particularly when the sample size is limited.

EXPERIMENTAL

All reagents and apparatus were checked and found to be free from interferences.

Reagents

The benzodiazepines and metabolites studied were diazepam, N-desmethyldiazepam, medazepam, flurazepam, N-desalkylflurazepam, N-hydroxyethylflurazepam, flunitrazepam, N-desmethylflunitrazepam, chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, nitrazepam, clonazepam, bromazepam (Roche, Welwyn Garden City, U.K.), oxazepam, lorazepam (Wyeth, Maidenhead, U.K.), ketazolam (Beecham, Brentford, U.K.), triazolam, alprazolam (Upjohn, Crawley, U.K.), pinazepam (Zambeletti, Milan, Italy), tetrazepam (Clin. Midy. International, Montpelier, France), lormetazepam (Schering, Burgess Hill, U.K.), clobazam, N-desmethylclobazam (Hoechst, Hounslow, U.K.), temazepam (Carlo Erba, Milan, Italy), and prazepam (Warner, Pontypool, U.K.).

All benzodiazepines were free bases except flurazepam and chlordiazepoxide which were supplied as their hydrochloride salts. These two compounds were converted to free bases by extraction from alkaline (pH 14) solution with redistilled ether for flurazepam and ethyl acetate for chlordiazepoxide. The organic extracts were washed with water, dried over saturated brine and magnesium sulphate, centrifuged and evaporated to dryness under nitrogen.

N,O-Bis(trimethylsilyl)acetamide (BSA) was supplied by Pierce (Chester, U.K.).

All solvents used were pesticide grade (Fisons, Loughborough, U.K.).

Amberlite XAD-7 porous polymer beads (20–50 mesh) (BDH, Poole, U.K.) were cleaned as described previously[31]. The beads were deactivated by treatment

TABLE I

PUBLISHED VALUES OF PEAK BLOOD CONCENTRATIONS AND ELIMINATION HALF-LIVES OF BENZODIAZEPINES IN A MAN AFTER A SINGLE SUBACUTE DOSE

Compounds are listed in the retention order given in Table II.

Benzodiazepine	*Ref.*	*Dose (mg)*	*Metabolites*	*Mean peak blood (B) serum (S) or plasma (P) concentration (ng/ml)*	*Mean time of peak blood concentration (h)*	*Mean elimination half-life (h)*
Medazepam**	1,2	30		141 (B)	1.5	2
			Diazepam	23	1 24	NR*
			N-desmethyldiazepam	28–195	84	NR
			N-desmethylmedazepam	79	1.5	NR
Oxazepam	3,4	45		1089 (P)	2	3.9, 8
			Oxazepam-C-glucuronide	999	3.4	5.6
Lorazepam	5	2		23 (P)	2.57	15.87
			Lorazepam-C-glucuronide	32.4	6.24	18.5
Diazepam	3	10		167 (B)	1.1	Mean 32, range 21–37
			N-Desmethyldiazepam	32	39	50–99
Ketazolam**	6	30		4 (P)	2	1.5
			Diazepam	17	10	28
			N-desmethyldiazepam and/or N-desmethylketazolam	127	14	50
Chlorazepate	3	15		66 (P)	0.9	2
			N-Desmethyldiazepam	230	1	64.8
Pinazepam	7	10		36.8 (P)	1.8	15.7
			N-Desmethyldiazepam	150	3	NR
Clobazam	3,8	20		465 (P)	1.7	24.5
			N-Desmethylclobazam	88	45.6	Mean 42, range 36–46
Flunitrazepam***	3,9,10	2		8 (P)	2	Mean 15, range 20–35
			N-Desmethylflunitrazepam	1.5	12	31

Bromazepam	3	12		131 (B)	1–4	11.9
Prazepam	3	30		6.6 (P)	0.5	1.3
			N-Desmethyldiazepam	321	4.25	96
Temazepam	3	20		668 (P)	0.75	8.3
			Temazepam-C-glucuronide	NR	–	1.9
Lormetazepam	11	1		6.3 (P)	2.2	9.9
			Lormetazepam-C-glucuronide	34	6	12–13
Flurazepam	3,12	60		1–5 (P)	0.5–1	3.1
			N-Desalkylflurazepam	30–50	3	Mean 65, range 32–100
			N-hydroxyethylflurazepam N-hydroxyethylflurazepam-C-glucuronide	150–250	1–4	3
Nitrazepam	13	5		40 (S)	2.3	29
Clonazepam	14	2		10.4 (B)	2.6	26.4
Chlordiazepoxide**	1,3,15,16	20		978 (P)	3.3	13.2
			N-Desmethylchlordiazepoxide	338	16	10–18
			Demoxepam	50 (S)	24	45
Alprazolam	17	1		13.4 (P)	0.7–1.6	12–15
			7α-Hydroxyalprazolam	NR	–	–
Triazolam	18	0.88		8.8 (P)	1.3	2.3
			7α-Hydroxytriazolam-C-glucuronide	6.1	1.3	3.9
			4α-Hydroxytriazolam-C-glucuronide	6.1	2.5	3.8

* NR = not reported.
** Limited studies only have been reported for these compounds.
*** Subjects were receiving additional medication.

with excess ethereal diazomethane for 2 h at room temperature. They were then rinsed with ether and stored under ether at 4°C.

All glassware, which was silanised as described previously[31], was rinsed with ether and dried with a stream of nitrogen immediately before use.

Stock solutions of benzodiazepines

Solutions of benzodiazepines or metabolites in methanol were stored in pyrex volumetric flasks at 4°C in the dark[32]. Some solutions *e.g.*, N-desmethylchlordiazepoxide were found to be unstable and so all were regularly monitored for evidence of decomposition. Stock solutions were diluted with toluene for direct injection or with water before adding to blood.

Benzodiazepines in blood: standard solutions, spiked samples, internal standards

Drug-free whole blood from a volunteer (JMFD) was treated with ethylenediaminetetraacetic acid disodium salt and stored in 1oz glass screw-cap vials (Aimer Products, London, U.K.) at 4°C. Standards were prepared by adding small volumes of dilute aqueous solutions of benzodiazepines, vortexing and allowing to equilibrate for at least 15 min before use. An identical procedure was used to prepare spiked samples or to add an internal standard to unknown samples.

Storage of blood samples

Most casework blood samples were as received from police surgeons either in 1oz glass screw-cap vials as described above or in 5 ml "Neutral Glass Vials" (Johnson and Jorgensen, London, U.K.) crimp sealed with synthetic rubber septa (W1888 Red Rubber, West Pharmarubber, St. Austell, U.K.). The blood contained potassium oxalate as anticoagulant and sodium fluoride as antibacterial agent. Several casework blood samples were submitted in hard polypropylene–polystyrene vials (30-ml Universal Container; Sterilin, Teddington, U.K.). All samples were partially or completely haemolyzed as a result of their treatment prior to receipt. They were subsequently stored at 4°C.

Sample preparation

Care must be taken during sample preparation to avoid contamination by plasticizers since they interfere with the analysis.

Toluene extraction. Internal standard, if required, was added to the blood before extraction. Blood (0.2 ml), pH 9.2 buffer (Gurr, BDH; 0.1 ml), and toluene (1.5 ml) were placed in a 10-ml centrifuge tube, vortexed for 30 sec, centrifuged and the organic layer pipetted into a 3.5-ml volume vial with an aluminium-foil-lined screw cap (FBG-Trident, Avon, U.K.). The extraction was repeated and the combined extracts evaporated under nitrogen at 60°C.

When 1-ml blood samples were analysed, 0.3 ml of buffer and a total of 8 ml of toluene were used for the extraction.

XAD-7 clean-up: qualitative analysis. The residue from the toluene extraction was dissolved in 20 μl of ether. Pentane (1 ml) was then added and the solution thoroughly mixed. Dry deactivated Amberlite XAD-7 porous polymer beads (1 mg; weighed out in an unsilanised glass container) were immediately added and the vial was gently rolled for 10 min on a mechanical rocker (Grant Instruments, Cambridge,

U.K.). The pentane solution was aspirated and the beads and the interior of the vial were thoroughly rinsed with a further three 1-ml portions of pentane. Residual traces of pentane need not be removed. The beads were thoroughly extracted with three 100-μl portions of ethyl acetate and the combined extracts evaporated under nitrogen. The residue was dissolved in 10 μl of toluene for analysis by GC–ECD.

XAD-7 clean-up: quantitative analysis. For quantitative analysis, the procedure for qualitative analysis was used with the quantities of Amberlite XAD-7 porous polymer beads and the volumes of toluene given in Table IV.

Derivatisation of temazepam and lormetazepam for quantitative analysis

A 50-μl volume of a 3% solution of BSA in acetone was added to the cleaned extract and the mixture evaporated at 60°C for 10 min under a stream of nitrogen. The residue was dissolved in 30 μl of toluene for analysis by GC–ECD.

Gas chromatography

Carlo Erba Model 4160 and 2150 gas chromatographs equipped with Model HT-25 ^{63}Ni electron-capture detectors and Model 251 electron-capture detector control modules were used. The Grob split/splitless injector fitted with a 0.25 ml silica injection port liner was used in the splitless mode with no septum purge or purge of the injection port after injection. Silica injection port liners were silanized as described below. The liners were cleaned as previously described[31] at intervals of 1–7 days depending on the amount of use.

Flexible fused-silica capillary columns (11 25 m × 0.25 mm I.D.; Packard Becker, Caversham, U.K.), were vapour-phase deactivated with three 0.3-μl injections of 1,3-diphenyl-1,1,3,3-tetramethyl disilazane (Fluka, Glossop, U.K.) followed by two 1-μl injections of methanol. The injections were made at 10 min intervals with a carrier gas flow-rate of 1 ml/min. The columns were then dynamically coated[31] using 4% SE-52 in toluene. Some columns were not deactivated before coating. Removal of the first 45 cm of the column may be necessary at intervals if deterioration of the peak shape and responses of the more polar benzodiazepines (*e.g.* nitrazepam) is observed.

The following conditions were used. Carrier gas: helium; flow-rate, 5.8 ml/min (25°C). Make-up gas: 5% methane in argon (Air Products, Southampton, U.K.); flow-rate, 45 ml/min (25°C), pressure 2.1 kg/cm^2. Injection port temperature, 250°C. Detector temperature, 300°C. Temperature programme: 130°C programmed at 30°C/min to 260°C; held at 260°C for 4 min, extra cool down time, 2 min.

The electron-capture detector was operated in the constant current mode with a potential of 50 V, a 1 μsec pulse width and a standing current of 2.8 nA.

A 1-μl Hamilton 7001N wire in needle syringe (Phase Separations), a Hamilton Syringe cleaner (Phase Separations), rubber septa (Carlo Erba) and a septum needle guide (Phase Separation) were used. The smaller aperture of the stainless steel injection port insert was placed uppermost. Injections were performed with a rapid depression of the plunger and a syringe-needle dwell time of 6 sec in the injection port to permit complete evaporation of the less volatile benzodiazepines.

Retention times were measured with a chart recorder (Model BD8, Kipp and Zonen, Delft, The Netherlands) at a chart speed of 5 cm/min or a Servoscribe pen recorder at a chart speed of 6 cm/min.

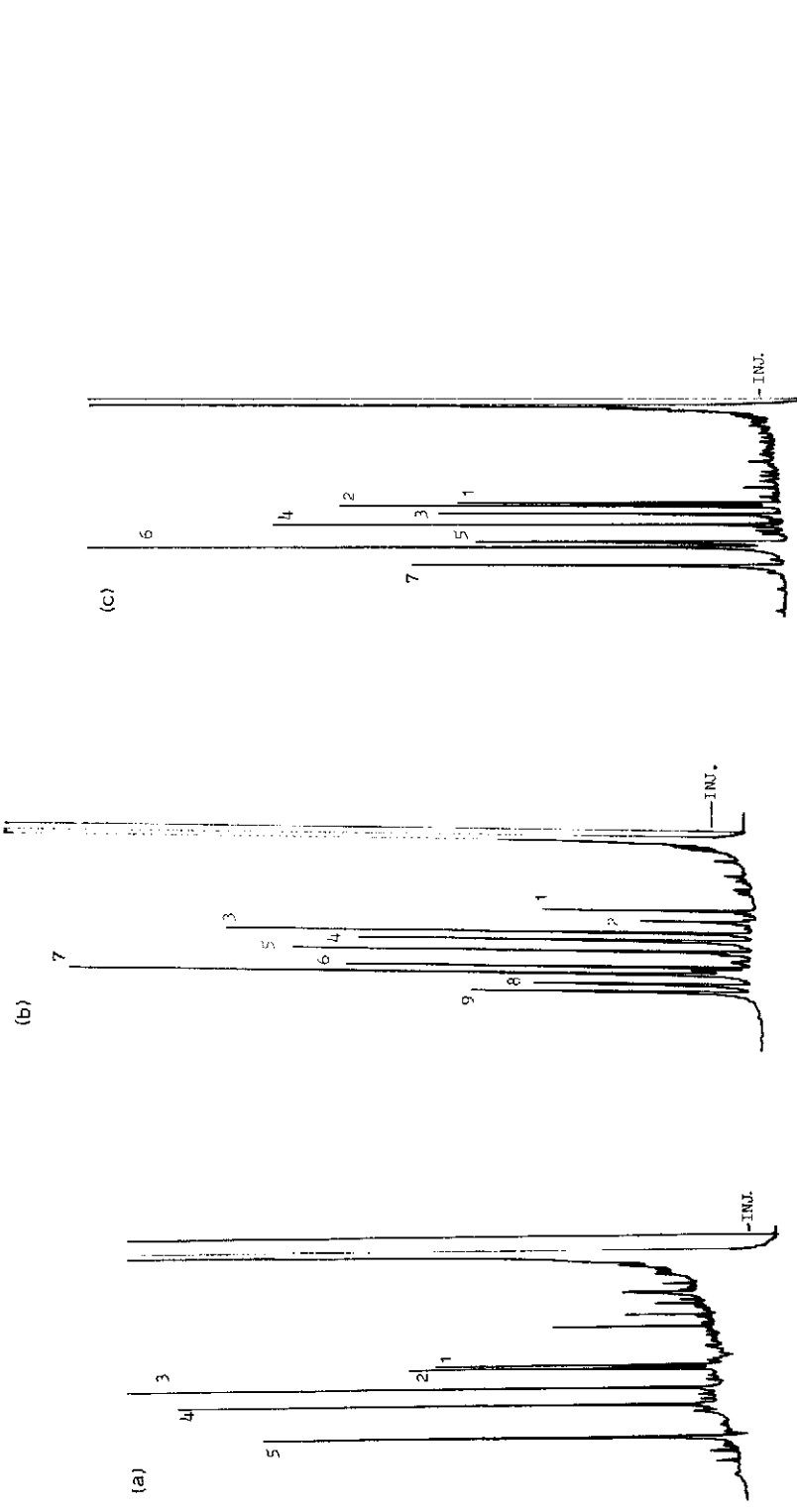


Fig. 2. Analysis of benzodiazepines on a deactivated SE-52 silica capillary column; conditions as described in the experimental section. (a) New 25 m × 0.25 mm I.D. column. Extract of 0.2 ml of blood spiked with 50 ng/ml each of lorazepam (1), diazepam (2), flunitrazepam (3), nitrazepam (4), and triazolam (5). (b) Same column after 8 months continual use analysing blood extracts. Mixture of benzodiazepines containing 1.6 ng of medazepam (1), 240 pg of oxazepam (2), 80 pg each of diazepam (3), N-desmethyldiazepam (4), flunitrazepam (5) and flurazepam (6), 1.2 ng of chlordiazepoxide (7) and 80 pg each of alprazolam (8) and triazolam (9). (c) Same column after 12 months continual use analysing blood extracts. Mixture of benzodiazepines containing 200 pg of lorazepam (1), 100 pg each of diazepam (2) and N-desmethyldiazepam (3), 50 pg of flunitrazepam (4), 150 pg of nitrazepam (5), 1.5 ng of chlordiazepoxide (6), and 150 pg of triazolam (7).

A 1-μl volume of a toluene solution containing 100 pg/μl each of lorazepam, flunitrazepam, nitrazepam and triazolam in toluene was analysed daily to check the performance of the system.

Qualitative analysis. Aliquots (1 μl) of the extracts were analysed by GC–ECD using a deactivated SE-52 capillary column. Retention times relative to medazepam were used to identify the separated constituents. It may be necessary to repeat the analysis on an un-deactivated column to identify compounds that are inadequately resolved by the deactivated column (Table II).

Quantitative analysis. Appropriate ranges of standards in blood were extracted and treated with Amberlite XAD-7 beads in an identical manner to the unknown samples. Aliquots (1 μl) of the extracts were analysed by GC–ECD on a deactivated SE-52 capillary column (25 m × 0.25 mm I.D.) using flunitrazepam, oxazepam and lormetazepam as internal standards (Table V). Peak-height ratios of analyte to internal standard were plotted against concentration.

Temazepam and lormetazepam were analysed as their O-trimethylsilyl ethers[26] while all the other benzodiazepines and metabolites (Table II) need not be derivatised before analysis.

RESULTS AND DISCUSSION

Sample preparation

When benzodiazepines and metabolites were analysed in concentrated toluene[25] extracts of blood, the injection port and column were contaminated with an active deposit which partially adsorbed polar benzodiazepines such as nitrazepam and caused a rapid deterioration of the column and the detector performance. Back extraction of the toluene extracts into acid gave unacceptable blank backgrounds. In addition some benzodiazepines (*e.g.* alprazolam and nitrazepam) are readily hydrolysed under acidic conditions and so this approach was not pursued.

A clean-up method previously developed for explosives analysis[31] was found, after some modifications, to be suitable for the trace analysis of low therapeutic levels of benzodiazepines in blood. The method involved selective extraction of polar compounds from solutions of biological extracts in pentane using Amberlite XAD-7 porous polymer beads. After thorough washing with pentane to remove unadsorbed coextractives and lipids, the compounds of interest were recovered from the beads with a polar solvent such as ethyl acetate. Repeated analysis of extracts prepared by this method gave acceptable blank backgrounds with no adverse effect on the column or detector performance.

The principle modification of the original method[31] involved deactivation of the polymer beads with diazomethane to prevent irreversible adsorption of basic metabolites. In addition a small volume of ether was used to dissolve the dry toluene extract before the addition of pentane in order to reduce adsorption of polar benzodiazepines such as lorazepam on to the surface of the silanised glass container.

The cleanest extracts were obtained with 1 mg of beads and so this weight was used in qualitative analysis at high sensitivity to demonstrate the presence or absence of benzodiazepines. The optimum weight of beads for quantitative analysis was 4–5 mg which resulted in good recoveries of all but the least polar benzodiazepines (Table III) and minimal interference by co-extractives.

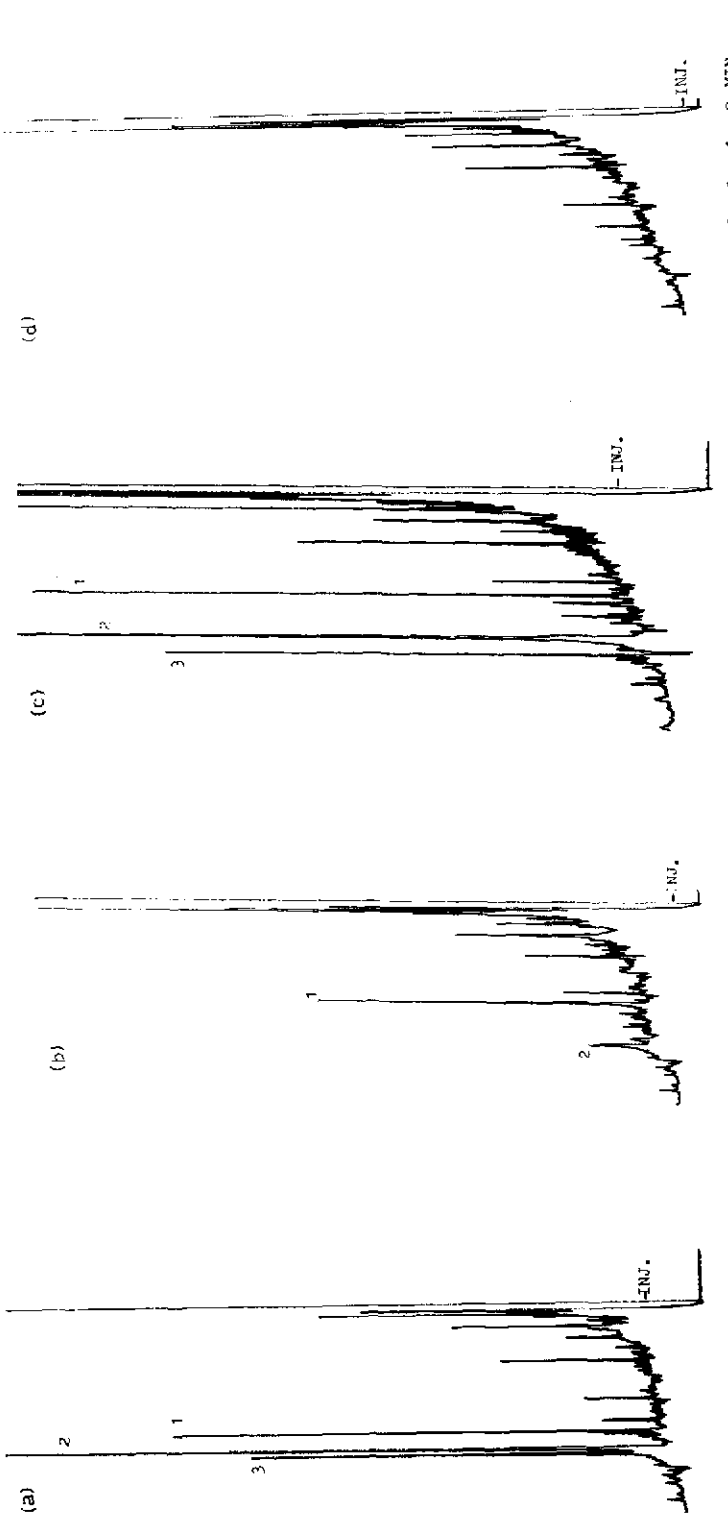


Fig. 3. Analysis of 0.2 ml blood samples spiked with low therapeutic levels of various benzodiazepines; conditions as described in the experimental section. (a) Blood spiked with 10 ng/ml of flunitrazepam (1), 30 ng/ml of nitrazepam (2), and 100 ng/ml of chlordiazepoxide (3). (b) Blood spiked at a level of 40 ng/ml of lorazepam (2). Peak (1) is lorazepam quinazoline-3-carboxaldehyde. Peak (2) is believed to be intact lorazepam. (c) Blood spiked at a level of 25 ng/ml of diazepam (1) and 10 ng/ml each of clonazepam (2) and triazolam (3). (d) Extract of 0.2 ml of blank blood. The small peaks are believed to be due to traces of plasticizers.

Amberlite XAD-4 and XAD-12 porous polymers were briefly tested in the clean-up method but the results were inferior to those obtained with Amberlite XAD-7.

Chromatography

All the benzodiazepines and metabolites in Table II were analysed with good peak shape and sensitivity by GC–ECD using SE-52 silica capillary columns (Figs. 2–5). Splitless injection was used with high flow-rates of carrier gas and a fast temperature programme rate to give optimum response for the less volatile benzodiazepines such as triazolam. Toluene was used as injection solvent in preference to more polar solvents in order to obtain maximum response for low levels of the more polar benzodiazepines[34].

Fifteen benzodiazepines and metabolites were resolved on a deactivated SE-52 column (25 m × 0.25 mm I.D.) and twenty two out of the twenty-six compounds described in this paper when both deactivated and un-deactivated SE-52 columns

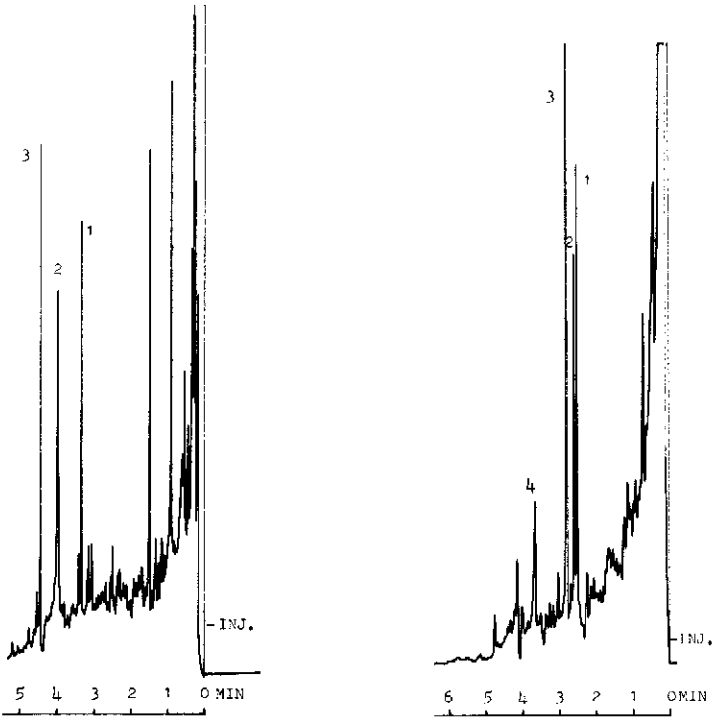


Fig. 4. Enhancement of sensitivity by increasing sample size. Extract of 1 ml of blood spiked with 2 ng/ml each of flunitrazepam (1), clonazepam (2), and triazolam (3); conditions as described in the experimental section.

Fig. 5. Analysis of 0.2 ml of blood from a driver who had been involved in a traffic accident. The sample was stored in a hard polystyrene–polypropylene vial (Sterilin, 30-ml Universal Container). Conditions as described in the experimental section. Peak identities: lorazepam quinazoline-3-carboxaldehyde corresponding to a therapeutic level of lorazepam (1), traces of diazepam (2) and N-desmethyldiazepam (3). Peak 4 is believed to be intact lorazepam.

TABLE II

CHARACTERISTICS OF THE GC ANALYSIS OF BENZODIAZEPINES AND THE TRACE ANALYSIS OF BENZODIAZEPINES IN BLOOD

Benzodiazepine	*Retention time (min)*		*Minimum detectable level (MDL)*		
	Deactivated SE-52 column (16 m × 0.25 mm I.D.)	*Un-deactivated SE-52 column (25 m × 0.25 mm I.D.)*	*Pure compound (pg)*	*MDL in 0.2 ml of blood (ng/ml)*	*MDL in 1 ml of blood (ng/ml)*
Medazepam	2.18	–	1000	500	–
Oxazepam*	2.46	–	20	20	–
Lorazepam*	2.67	–	10	5	2
Diazepam	2.77	–	5	1	–
Tetrazepam	2.77	–	10	1	–
Ketazolam**	–	–	10	0.5**	–
N-Desalkylflurazepam	2.83	–	5	1	0.5
N-Desmethyldiazepam	2.98	–	5	1	–
Pinazepam	3.05	–	10	2	–
Clobazam	3.12	–	20	3	–
Flunitrazepam	3.27	–	1	1	0.2
Prazepam	3.35	4.24	5	1	–
N-Desmethylclobazam	3.35	4.33	20	10	–
Bromazepam	3.35	4.33	20	10	
Temazepam***	3.23,3.46	–	10	5	–
N-Hydroxyethylflurazepam	3.46	–	30	2	
Lormetazepam***	3.46,3.55	–	20	3	0.2

N-Desmethylflunitrazepam	3.55	–	20	1	–
Flurazepam	3.67	4.56	5	1	–
Nitrazepam	3.69	4.61	5	1	–
Clonazepam	3.88	4.80	1	1	0.2
Chlordiazepoxide	3.88	5.01	1000	50	–
N-Desmethylchlordiazepoxide	3.88	5.07	250	50	–
Demoxepam[§]	3.93	5.01[§§]	1000	50	–
Alprazolam	4.13	–	20	2	1
Triazolam	4.35	–	10	1	0.2
4-α-Hydroxytriazolam	4.92	–	–	–	–

* High retention time peaks were observed when the compounds were analysed at high levels using a clean system (lorazepam t_R 3.88 min and oxazepam t_R 3.53 and 3.78 min).

** Breaks down quantitatively to diazepam[33]. Enhanced sensitivity due to higher recovery of ketazolam compared with diazepam.

*** Breakdown of underivatised compounds occurs. Temazepam-O-TMS: t_R = 4.18 min; lormetazepam-O-TMS: t_R = 4.38 min using conditions described in the experimental section but holding the initial temperature for 1 min before programming.

§ Injection port temperature 180°C. At a temperature of 250°C partial decomposition to N-desmethyldiazepam occurs (MDL 5 ng/ml of demoxepam in blood).

§§ t_R 4.92 min on new column rapidly changing on use to t_R 5.01 min.

TABLE III

REPRODUCIBILITY OF ANALYSIS AND RECOVERY OF COMMONLY ENCOUNTERED BENZODIAZEPINES

The compounds tested are representative of the various chemical types of benzodiazepines.

Benzodiazepine	*Reproducibility of analysis of pure benzodiazepines in toluene*		*Reproducibility of recovery of benzodiazepines from 0.2 ml of blood*	
	R.S.D. % (n = 10)	*pg Injected*	*Mean recovery % (R.S.D. %; n = 5)*	*Spiked blood level (ng/ml)*
Lorazepam	7.8	200	95 (9.7)	40
Diazepam	3.0	100	56 (2.6)	50
N-Desalkylflurazepam	3.5	100	88 (3.4)	25
N-Desmethyldiazepam	3.3	100	81 (3.9)	25
Flunitrazepam	1.5	50	71 (5.1)	10
Temazepam*	1.2	100	61 (9.8)	50
Nitrazepam	2.9	100	70 (5.1)**	25
Chlordiazepoxide	3.8***	2000	66 (9.9)	200
Triazolam	3.0***	100	79 (5.5)**	10

* Analysed as the O-TMS ether.
** $n = 3$.
*** $n = 5$.

were used (Table II). The un-deactivated columns gave superior resolution of some longer retained benzodiazepines but in practice slight retention time variations were observed possibly due to progressive deactivation of the column by blood extracts. A deactivated column was therefore used routinely for both qualitative and quantitative analysis while an un-deactivated column was occasionally used as a second column in qualitative analysis only. Polar stationary phases such as XE-60 were unsatisfactory because the more polar compounds such as triazolam were not eluted. Retention time reproducibility was determined for the representative range of benzodiazepines shown in Table III, and found to be satisfactory; relative standard deviation (R.S.D.) 0.4% ($N = 10$).

The minimum detectable levels of benzodiazepines were in the low picogram range except for medazepam[33], chlordiazepoxide, and demoxepam where poor electron capturing properties or thermal instability limited the sensitivity to the low nanogram range (Table II). The reproducibility of response of a representative range of benzodiazepines was determined at nanogram and low picogram levels and found to be satisfactory (Table III). It is important to use the injection technique described in the experimental section in order to obtain optimum sensitivity and reproducibility.

Routine analysis of samples did not significantly degrade the performance of the column or the detector over a one year period. Regular cleaning of the injection port liner as described in the experimental section was usually sufficient to maintain the performance of the system. Removal of the first 45 cm of the column restores the peak shape and reproducibility of response of the more polar benzodiazepines when these deteriorate in the long term. The qualitative results in this paper (Figs. 2–5) were obtained from a single column after 12 months of continuous use.

Qualitative analysis

The minimum detectable levels (MDLs) of the majority of benzodiazepines and metabolites in 0.2 ml blood samples were found to be in the low ng/ml range (Table II) with the result that only medazepam and ketazolam could not be detected at a single-dose therapeutic level. However, the medazepam metabolites (diazepam and N-desmethyldiazepam) are readily detected and ketazolam decomposes quantitatively on injection to give diazepam which is readily detected.

The response and MDLs of benzodiazepines of high milligramme potency can be improved through the use of larger blood volumes if available (Table II; Fig. 4). Contamination of the blood sample by plasticizers or storage at room temperature for extended periods gives high blank backgrounds. This problem can be minimised by storing the sample as described in the experimental section. Co-extractives in badly deteriorated post-mortem bloods sometimes obscure areas of the chromatogram, but many benzodiazepines may still be detected in these samples by the method described in this paper (Fig. 6). Drugs other than benzodiazepines may be encountered in forensic samples but can usually be distinguished from benzodiazepines on the basis of retention time.

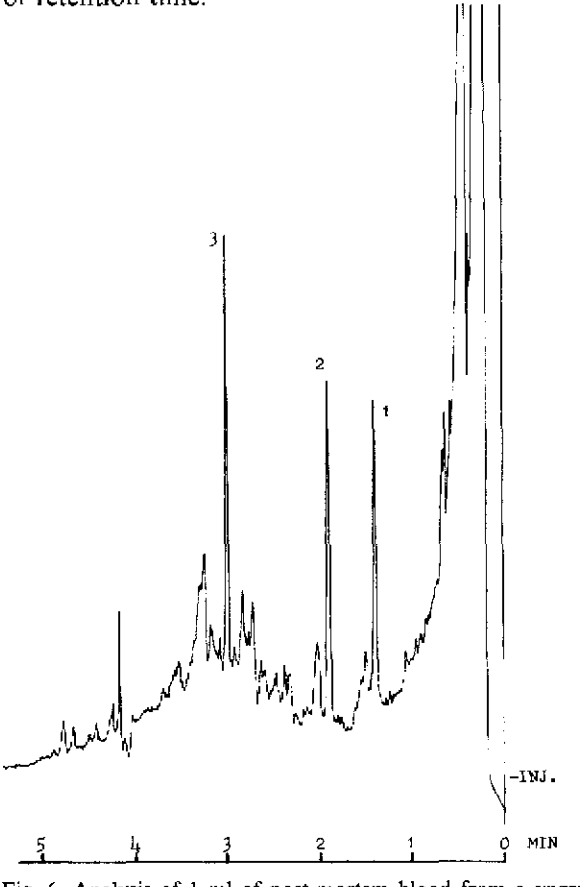


Fig. 6. Analysis of 1 ml of post-mortem blood from a vagrant (cause of death: inhalation of vomit). Conditions as described in the experimental section with a chart speed of 20 mm/min. Peak identities: co-extractive (1), medazepam internal standard (2) and approximately 1 ng/ml flunitrazepam (3).

TABLE IV

QUANTITATIVE ANALYSIS: SAMPLE PREPARATION AND LINEAR RANGE

Benzodiazepine	*Investigated linear range (ng/ml)*	*Volume of toluene used to dissolve final extract (μl)*	*Mass of XAD-7 (mg)*
Lorazepam	0 80	30	5
Diazepam	0–100	60	5
N-Desalkylflurazepam	0–50	60	5
N-Desmethyldiazepam	0–50	60	5
Temazepam	0–50	30	4
Nitrazepam	0 75	60	4
Chlordiazepoxide	0–500	30	4
Triazolam	0 25	25	4
Triazolam	0–2.5*	30	1

* Result obtained using 1 ml blood samples.

The qualitative analytical method has been used extensively to identify benzodiazepines present at very low levels in blood samples. In these cases, positive benzodiazepine RIA results[24] were obtained but could not be confirmed by packed column GC–ECD. For example flunitrazepam was identified in 0.2 ml aliquots of blood samples from a number of robbery victims. This compound, which has a high milligramme potency (Table I), had been administered as an incapacitating agent in combination with alcohol to the unsuspecting victims. The method has also found much use in the detection of chlordiazepoxide and its metabolites in small blood samples, since these compounds are thermally unstable and cannot be analysed adequately by packed-column GC–ECD. A typical drugs/driving case where the driver was involved in an accident while under the influence of benzodiazepines is shown in Fig. 5. A typical post-mortem case of a vagrant who died through inhalation of vomit while under the influence of benzodiazepines and alcohol is shown in Fig. 6.

TABLE V

ACCURACY AND PRECISION OF ANALYSIS DETERMINED BY BLIND TRIAL

Benzodiazepine	*Blood level (ng/ml)*			*Internal standard*
	Spiked	*Found*	*R.S.D. % (n = 3)*	
Lorazepam	20	21.5	3.9	Oxazepam
Diazepam	50	53	1.6	Flunitrazepam
N-Desalkylflurazepam	25	27.5	2.7	Flunitrazepam
N-Desmethyldiazepam	25	27.5	2.8	Flunitrazepam
Temazepam	25	23.0	2.0	Lormetazepam
Nitrazepam	20	20.0	5.4	Flunitrazepam
Chlordiazepoxide	100	99.0	11.1	Oxazepam
Triazolam	8	7.7	5.5	Flunitrazepam

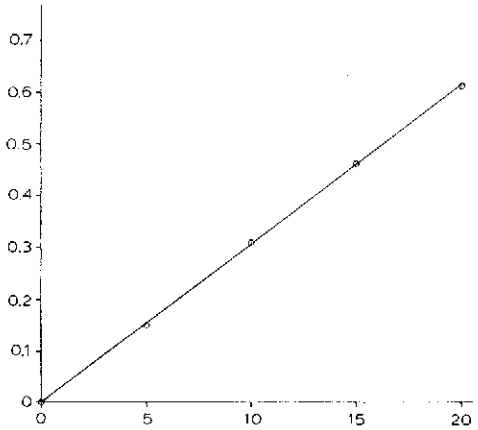


Fig. 7. Typical calibration graph for the quantitative analysis of triazolam in 0.2 ml blood samples in the range 0–20 ng/ml using flunitrazepam as internal standard. ($Y = 0.03X - 0.01$; $r = 0.9998$). A single analysis was used for each point on the graph. *X*-Axis: Concentration of triazolam in blood (ng/ml). *Y*-Axis: Ratio of triazolam peak height to flunitrazepam peak height.

Quantitative analysis

The quantitative aspects of the technique were studied for a representative group of nine of the most commonly encountered benzodiazepines (Tables III–V).

Calibration plots of benzodiazepine–internal standard ratios *vs.* concentration were shown to be linear over the ranges shown in Table IV. A typical calibration plot for triazolam is shown in Fig. 7. The accuracy and precision of the analysis and the reproducibility of recovery of the benzodiazepines at therapeutic levels in blood were found to be satisfactory (Tables III and V). The quantitative aspects of the method have been evaluated by a series of blind trials with good results (Table V).

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