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DETERMINATION OF DIAZEPAM AND ITS MAJOR METABOLITES IN MAN AND IN THE CAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of diazepam, and its major metabolites, oxazepam, temazepam and nordiazepam in plasma, blood, and urine of humans and cats. The assay for the compounds involves extraction into benzene—methylene chloride (90:10) from plasma, blood or urine buffered to pH 9.0. In both species the overall recovery of diazepam and its major metabolites from plasma or blood ranged from 60 ± 3.2 to 89 ± 13% (S.D.) and for urine from 79 ± 7.9 to 93 ± 10.5% (S.D.).

The sensitivity limit of the assay using UV detection at 254 nm was 50 ng/ml of plasma and blood in both species except for human urine (post-Glusulase) which was 200 ng/ml. The HPLC assay was used to monitor the plasma concentration—time profile in humans following a 10-mg oral dose of diazepam and the blood concentration time profile of diazepam and nordiazepam in cats following a 10 mg/kg intravenous dose of either diazepam or nordiazepam. The HPLC assay data correlated well with data generated by an electron-capture—gas—liquid chromatographic assay.

INTRODUCTION

The pharmacology of diazepam is well defined in man and many animal species [1—3]. Metabolic studies in man and animals [4] have shown that the compound is biotransformed to form three major metabolites (Fig. 1). Several attempts have been made to determine diazepam and its major metabolites simultaneously by electron-capture—gas—liquid chromatography (EC—GLC) [5—7], thin-layer chromatography (TLC) [8] and high-performance liquid chromatography (HPLC) [9, 10]. In general, the EC—GLC and TLC assays either could not separate diazepam and its three major metabolites simultaneously, were time consuming, needed a large sample (1.0 ml or greater),

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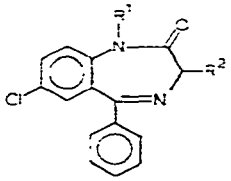

Compound	R^1	R^2
Diazepam	CH_3	H_2
Nordiazepam	H	H_2
Temazepam	CH_3	OH
Oxazepam	H	OH

Fig. 1. Chemical structures of diazepam and its metabolites.

and/or had a long analysis time making the assay impractical for routine use in pharmacokinetic studies where rapid sample throughput is very desirable.

The purpose of the study was to develop an analytical method suitable for pharmacokinetic studies capable of determining diazepam and its three major metabolites simultaneously using small samples of biological fluid. Previous studies in the cat [11] using ^{14}C-labelled diazepam indicated that hydroxylation was a major biotransformation pathway in this species and prompted further study.

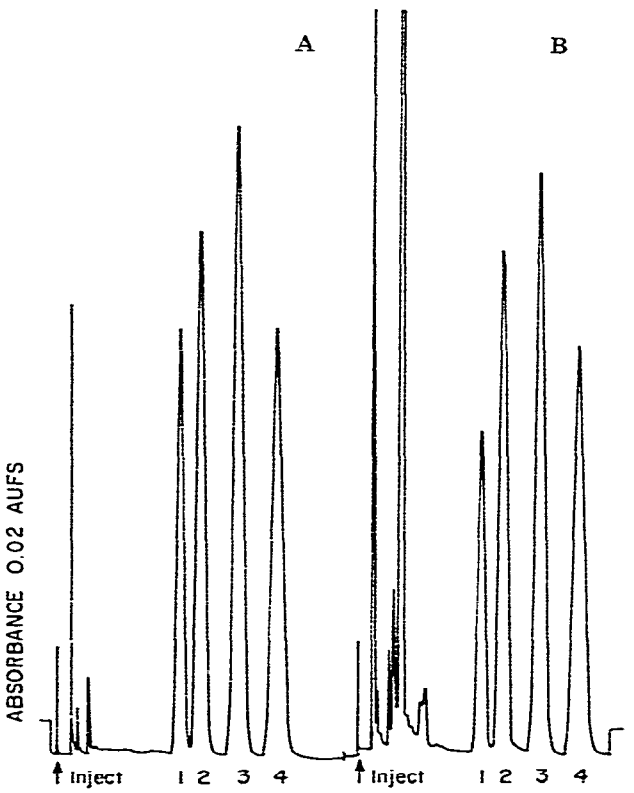

Fig. 2. Chromatograms of (A) standard mixture of 200 ng of each compound, and (B) human plasma containing 400 ng of each compound per ml taken through the extraction procedure. Peaks: 1 = oxazepam (k' = 6.0), 2 = temazepam (k' = 7.2, α = 1.2), 3 = nordiazepam (k' = 9.2, α = 1.3), 4 = diazepam (k' = 10.8, α = 1.2).

EXPERIMENTAL

HPLC parameters

Column parameters. The column used was a prepacked 30 cm × 3.95 mm I.D. stainless-steel column containing Bondapak (10 μm) C_{18} reversed-phase packing (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters. A Waters Assoc. high-performance liquid chromatograph Model ALC/GPC-204/6000A with a Model U6K injection system and a Model 440 UV detector with a 254 nm wavelength kit was used. All chromatograms were recorded on a 10-mV Hewlett-Packard recorder at a chart speed of 0.25 in./min.

The mobile phase used was methanol 550 ml diluted to 1000 ml with distilled deionized water. The solvent flow-rate was 2.0 ml/min.

Representative chromatograms of human plasma, human urine (pre- and post-Glusulase) and cat blood extracts, the retention times (t_R), capacity factors (k'), and the separation factor (α) of the compounds analyzed under the above conditions are given in Figs. 2, 3 and 4.

Analytical standards

All analytical standards were of pharmaceutical grade purity (> 99%). These included diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodia-

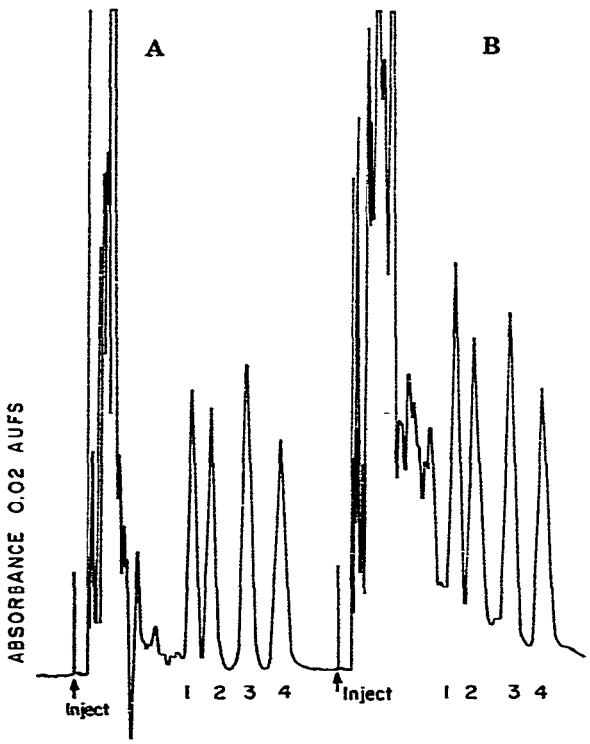

Fig. 3. Chromatograms of human urine containing 400 ng of each compound per ml, extracted (A) pre-Glusulase, and (B) post-Glusulase incubation. Peaks: 1 = oxazepam (k' = 8.0), 2 = temazepam (k' = 9.5, α = 1.2), 3 = nordiazepam (k' = 12.5, α = 1.3), 4 = diazepam (k' = 15.0, α = 1.2).

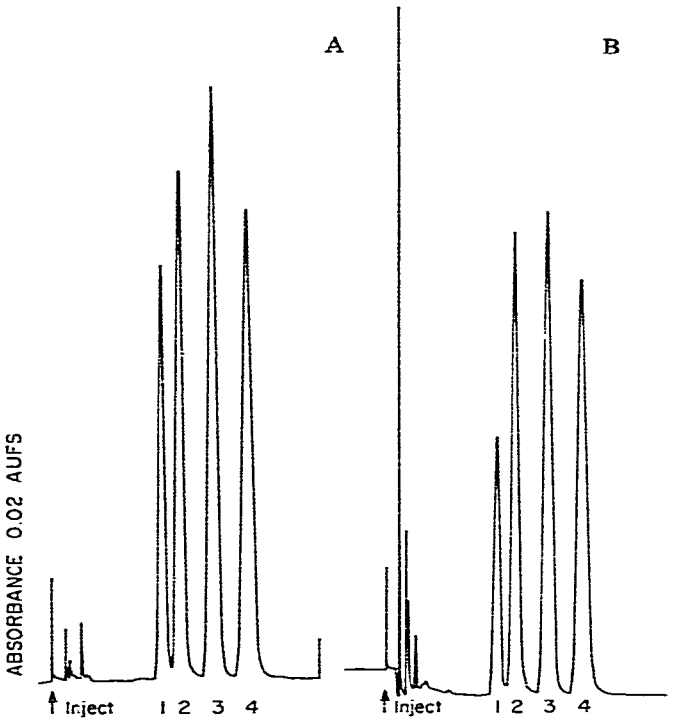

Fig. 4. Chromatograms of (A) standard mixture containing 200 ng of each compound, and (B) cat blood containing 400 ng of each compound per ml taken through the extraction procedure. Peaks: 1 = oxazepam (k' = 7.2), 2 = temazepam (k' = 8.3, α = 1.1), 3 = nordiazepam (k' = 11.0, α = 1.2), 4 = diazepam (k' = 13.8, α = 1.2).

zepin-2-one, $C_{16}H_{13}N_2OCl$, MW 284.74), nordiazepam (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{15}H_{10}N_2OCl$, MW 270.72), temazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{16}H_{13}N_2O_2Cl$, MW 300.74), and oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{15}H_{10}N_2O_2Cl$, MW 286.72).

Preparation of standard solutions

Weigh 10 mg of each of the compounds into a 100-ml volumetric flask and dissolve in 1 ml of ethanol. Dilute to 100 ml with benzene to yield a stock solution containing 100 μg/ml of each component. Dilute 1 ml of the stock solution to 100 ml with benzene to yield a working solution containing 1.0 μg/ml.

Aliquots of the respective working solutions (50—1000 μl) are transferred into separate 15-ml centrifuge tubes, evaporated to dryness under nitrogen at 60°C, and the residues are dissolved in 50 μl methanol to yield mixed standard solutions containing each of the four compounds in the amounts indicated

below:

Mixed standard solutions	Compound (ng/50 μl)
A	50
B	200
C	400
D	800
E	1000

Twenty-five microliters of the mixed standard solutions (A, B, C, D or E), are injected as external standards for establishing the HPLC parameters. Separate aliquots (50—1000 μl) of the working solution (1 μg/ml) are transferred into 15-ml centrifuge tubes, evaporated to dryness under nitrogen at 60°C, the residues are dissolved in an appropriate volume (0.1—1.0 ml) of plasma, blood or urine of either human or cat and are used as the processed standards to establish a calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery.

Reagents

All reagents used were of analytical reagent grade (> 99% purity). The inorganic reagents used are 1.0 *M* borate—Na_2CO_3—KCl buffer (pH 9.0) and 1.0 *M* phosphate buffer (pH 5.3) prepared as described [5].

The organic solvents used (nanograde purity) are methylene chloride and benzene (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

Calibration of diazepam and its major metabolites by HPLC

A calibration (external standard) curve of peak height of each component versus concentration in the range 25—500 ng per 25 μl injected is constructed. Calibration curves of both the external and the recovered standards were prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Analysis of human plasma or cat blood

Into a 15-ml centrifuge tube (PTFE No. 13 stoppered) add 0.5—1.0 ml of human plasma or 0.1—1.0 ml of cat blood, 0.2—1 ml of 1.0 *M* H_3BO_3—Na_2CO_3—KCl buffer (pH 9.0), and mix well on a Vortex action mixer. Extract the mixture with 5 ml of benzene—methylene chloride (90:10) by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes per min. Along with the samples, run six 1-ml specimens of control human plasma, urine, cat blood and urine. One used as a control blank and five containing 50 μl of standard solutions, A—E evaporated to dryness before adding the various biological fluids. Centrifuge the samples at 2200 rpm for 5 min (Damon/IEC Centrifuge Model PR-J, rotor No. 250 at 1300 *g*) and transfer a 4.8-ml aliquot of the upper organic layer into a 5-ml conical centrifuge tube. Evaporate the organic layer to dryness at 60°C in an N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean dry nitrogen. Dissolve the residue in a 50-μl aliquot of the mobile phase used in the

respective assay and inject a 25-μl aliquot for HPLC analysis.

Analysis of human or cat urine

Unconjugated fraction. One ml of human or cat urine is processed exactly as described for the analysis of human or cat blood. This fraction represents intact drug and unconjugated metabolites.

Conjugated fraction. The urine specimen remaining after the extraction of the unconjugated fraction is titrated with 1.0 *N* HCl to pH 5.3 and buffered with 1.0 *M* phosphate buffer. Add 2% Glusulase® enzyme preparation containing 150,000 units of glucuronidase and 100,000 units of sulfatase per ml (Endo Labs., Garden City, NY, U.S.A.) by volume of sample taken for analysis and incubate in a Dubnoff metabolic shaker at 37°C for 12—18 h (overnight). After cooling, the pH of sample is adjusted to 9.0 with 2 *N* NaOH and the sample extracted as described for plasma or blood.

Calculations

The concentration of each compound in the unknowns is determined by interpolation from the respective calibration curve of the recovered standards processed along with the unknowns, using the direct calibration (peak height versus concentration) technique. The percent recovery of each compound is determined by comparing the slope value (peak height per ng of compound) of the recovered standard curve to that of the external standard curve.

RESULTS AND DISCUSSION

The utility of EC—GLC in the analysis of diazepam and its major metabolites is well documented [5—7]. Although it is inherently more sensitive than HPLC analysis (with UV detection) for the determination of diazepam and nordiazepam per se, the analysis of temazepam and oxazepam requires silylation to the trimethylsilyl ether derivative, not only for enhanced sensitivity and chromatographic behavior, but also to ensure thermal stability, especially of oxazepam. The procedural details required make the simultaneous analysis of all four compounds by EC—GLC [5] a difficult procedure, at best.

The intrinsic UV absorbance of the benzodiazepines and their amenability to HPLC analysis, especially of thermally unstable compounds such as oxazepam or those that require derivatization prior to EC—GLC analysis (temazepam) have been used to advantage in pharmacokinetic studies [9, 12, 13].

Reversed-phase HPLC is preferred for the analysis of benzodiazepines since it provides better separation than does adsorption chromatography [12]. The pH of extraction and solvents used were selective for the quantitative extraction of the respective compounds of interest, evidenced by the absence of endogenous interfering peaks in the retention areas of diazepam and its major metabolites. All the compounds have sufficient UV absorbance at 254 nm for their accurate quantitation in the nanogram range.

The sensitivity of the assay for all four compounds in human plasma, cat blood, human urine (pre-Glusulase) and cat urine (both pre- and post-Glusulase

incubation) was 50 ng/ml. The sensitivity for all four compounds in human urine post-Glusulase, however, was 200 ng/ml.

Statistical validation

The overall mean recovery of diazepam and its major metabolites over the concentration range of 50—1500 ng/ml from various biological fluids is presented in Table I. The recovery of temazepam and nordiazepam was more consistent ranging from 81—89%, whereas that for diazepam (73—93%) and oxazepam (60—87%) showed a greater variation in the four biological fluids studied.

The intra-assay variability of diazepam and its metabolites recovered from human plasma, urine, cat blood and urine is presented in Table II. The mean intra-assay variability for all four compounds ranged from 4.2—6.9% over the concentration range of 50—1500 ng/ml.

TABLE I

OVERALL PERCENT RECOVERY (± S.D.) OF DIAZEPAM AND ITS MAJOR METABOLITES FROM VARIOUS BIOLOGICAL FLUIDS

	Concentration range (ng/ml)	Human plasma	Cat blood	Human* urine	Cat urine
Diazepam	50— 800	73 ± 5.0	74 ± 5.6	93 ± 10.5	79 ± 7.9
Nordiazepam	50— 800	84 ± 4.8	85 ± 8.0	85 ± 6.2	84 ± 12.1
Temazepam	50—1500	89 ± 13.0	81 ± 9.0	87 ± 11.3	88 ± 4.1
Oxazepam	50—1500	60 ± 3.2	63 ± 7.0	81 ± 10.4	87 ± 6.3

*The limit of quantitation for all four compounds in human urine (post-Glusulase) is 200 ng/ml.

TABLE II

INTRA-ASSAY VARIABILITY OF DIAZEPAM AND ITS MAJOR METABOLITES IN HUMAN PLASMA, URINE (PRE- AND POST-GLUSULASE), CAT BLOOD AND URINE (PRE- AND POST-GLUSULASE)

In all cases $n = 5$.

	Amount added (ng/ml)	Amount recovered (ng/ml)	Standard deviation (%)
Diazepam	50*	54	7.7
	50**	50	8.0
	50***	50	8.0
	50§	50	4.0
	50§,§§	50	4.0
	100*	97	4.9
	200**,§§	200	6.5
	300*	300	7.4
	300***	300	5.3
	300§	300	13.3
	300§,§§	300	7.3
	1500**	1500	8.5
	1500**,§§	1500	4.2
		Average	6.9

(Continued on p. 102)

TABLE II (continued)

	Amount added (ng/ml)	Amount recovered (ng/ml)	Standard deviation (%)
Temazepam	50*	55	2.0
	50**	50	6.0
	50***	50	8.0
	50§	50	0.0
	50§,§§	50	0.0
	100*	96	5.0
	200**,§§	195	4.1
	300*	301	6.7
	300***	300	4.7
	300§	300	10.0
	1500**	1500	5.5
	1500**,§§	1500	3.6
			Average 4.6
Nordiazepam	50*	54	0.0
	50**	50	2.0
	50***	50	8.0
	50§	50	0.0
	50§,§§	50	0.0
	100*	96	5.0
	200**,§§	200	4.2
	300*	300	7.4
	300***	300	4.7
	300§	300	9.3
	300§,§§	300	4.0
	1500**	1500	6.3
	1500**,§§	1500	3.5
			Average 4.2
Oxazepam	50*	51	4.3
	50**	50	9.0
	50***	50	4.3
	50§	50	8.9
	50§,§§	50	0.0
	100*	98	3.6
	200**,§§	200	4.3
	300*	300	6.9
	300***	300	2.6
	300§	300	13.3
	1500**	1500	7.9
	1500**,§§	1500	2.4
			Average 5.4

* In human plasma.
** In human urine.
***In cat blood.
§ In cat urine.
§§ Pre- and post-Glusulase.

The inter-assay variability of diazepam and its major metabolites recovered from human plasma and cat blood is presented in Table III. The mean inter-assay variability for all four compounds ranged from 6.2—8.4% over the con-

TABLE III

INTER-ASSAY VARIABILITY OF DIAZEPAM AND ITS MAJOR METABOLITES IN HUMAN PLASMA AND CAT BLOOD

	Amount added (ng/ml)	Amount recovered (ng/ml)	*n*	Standard deviation (%)	
Diazepam	50	54	5		11.0
	100	104	5		6.7
	100*	109	4		12.0
	150	154	5		14.9
	200	197	4		8.5
	200*	187	4		9.0
	300	303	4		2.9
	400*	394	3		5.3
	800*	789	3		4.9
				Average	8.4
Nordiazepam	50	51	6		15.0
	100	106	6		6.0
	100*	111	4		12.0
	200	197	6		4.1
	200*	185	4		3.7
	300	303	4		2.0
	800*	853	3		5.7
				Average	6.9
Temazepam	50	53	6		12.6
	100	102	6		5.4
	100*	103	4		1.3
	150	141	4		9.1
	200	196	6		7.1
	200*	191	4		13.0
	300	304	4		2.9
	400*	402	4		4.1
	800*	805	4		0.3
				Average	6.2
Oxazepam	50	51	6		12.8
	100	100	6		5.7
	100*	99	4		5.9
	150	147	4		6.3
	150*	150	3		16.0
	200	199	6		5.1
	200	195	4		8.4
	300	298	4		2.0
	800	817	2		2.3
				Average	7.2

*Data from cat blood.

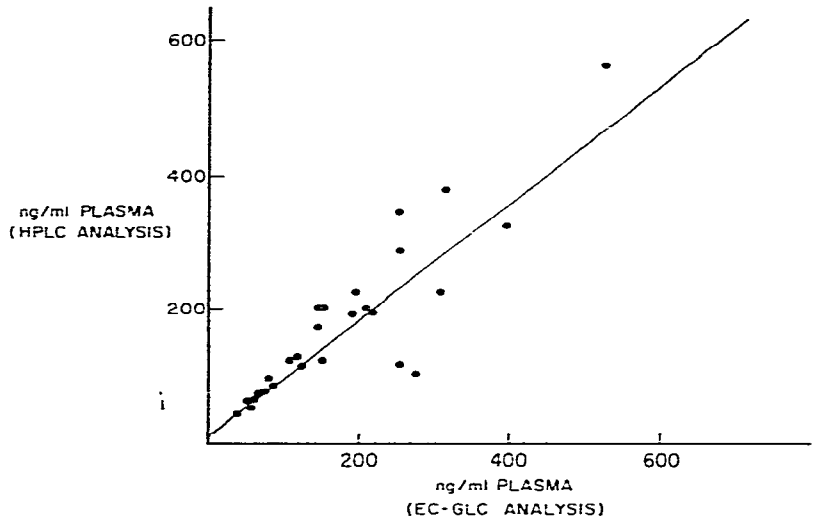

Fig. 5. Plasma concentrations (ng/ml) of diazepam in man following a single 10-mg oral dose, determined by HPLC vs. EC—GLC. $y = 0.938\,x + 15.9$; $r^2 = 0.831$, $r = 0.912$.

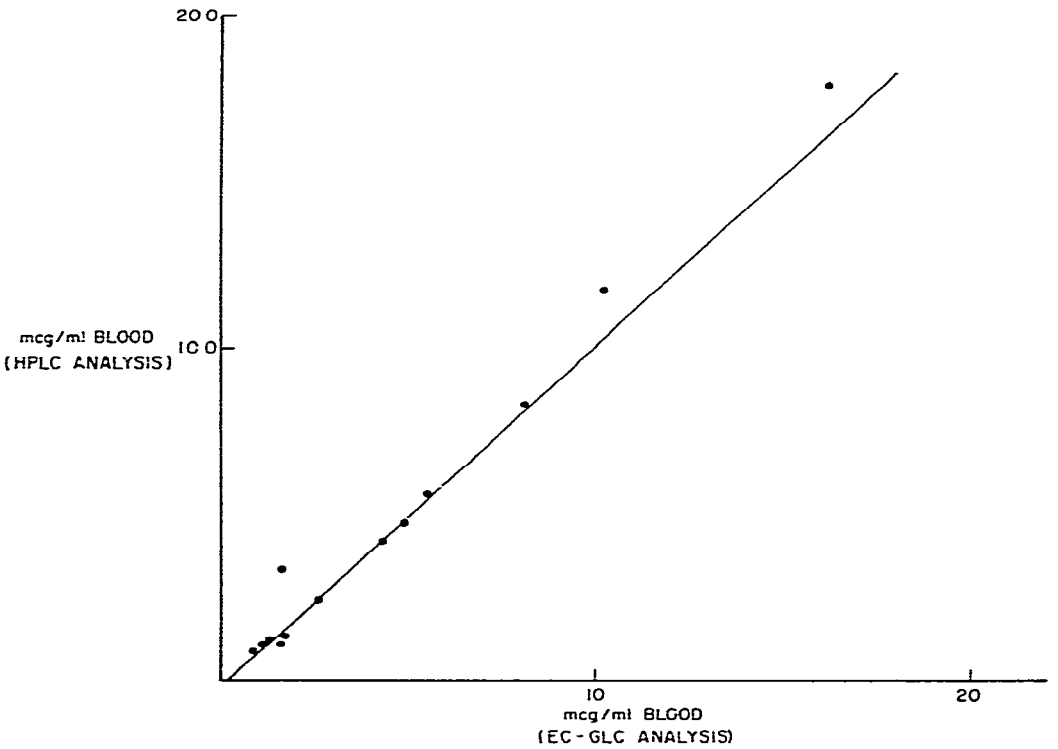

Fig. 6. Blood concentrations (μg/ml) of diazepam in a cat following a single intravenous dose of 10 mg/kg determined by HPLC vs. EC—GLC. $y = 1.10\,x - 0.17$; $r^2 = 0.985$, $r = 0.992$.

Application of the method in biological specimens

Human plasma and cat blood samples following diazepam administration, and cat blood samples following nordiazepam administration were analyzed by the HPLC assay method reported, and validated against the EC—GLC method previously reported [7].

A comparison of the data (HPLC vs. EC—GLC) from three human subjects, Fig. 5 (diazepam) and one cat, Fig. 6 (diazepam) and Fig. 7 (nordiazepam) determined by regression analysis showed a high degree of correlation (r = 0.912, 0.992, and 0.989, respectively) between the two methods, thus validating the clinical utility of the HPLC method for the analysis of biological samples.

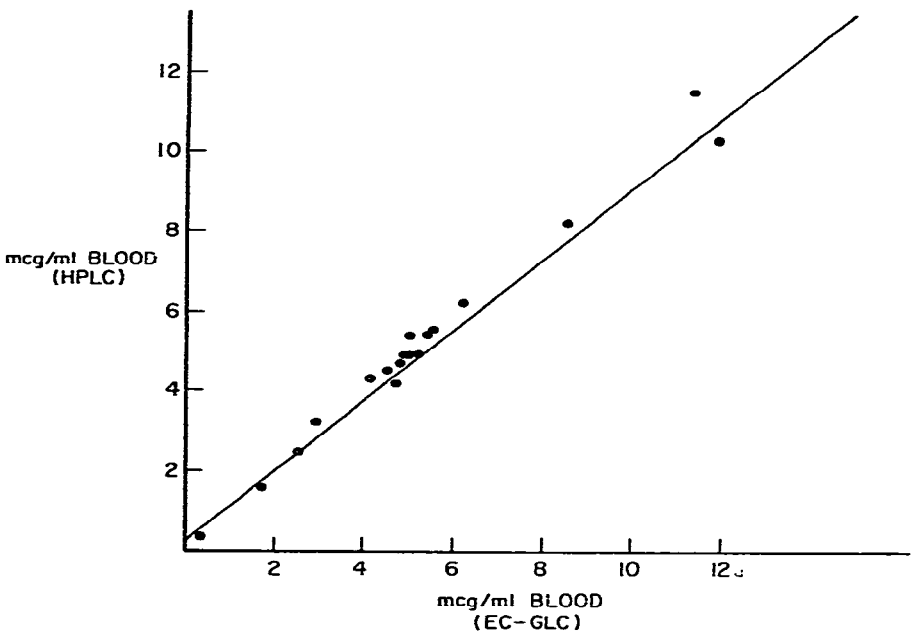

Fig. 7. Blood concentrations (μg/ml) of nordiazepam in a cat following a single intravenous dose of 10 mg/kg determined by HPLC vs. EC—GLC. $y = 0.924\ x + 0.300$; $r^2 = 0.978$, $r = 0.989$.

CONCLUSIONS

A sensitive and specific reversed-phase HPLC assay was developed for the determination of diazepam and its major metabolites in a variety of biological fluids: blood, plasma and urine. The method was applied to pharmacokinetic studies of diazepam in man and of diazepam and nordiazepam in the cat, and correlated well against the established EC—GLC assay, validating its clinical utility.

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