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DETERMINATION OF PINAZEPAM AND ITS METABOLITES IN SERUM, URINE AND BRAIN BY GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

A sensitive and specific assay, involving electron capture gas–liquid chromatography, has been developed for the identification of pinazepam and its metabolites in serum, urine and brain samples from dogs and rats after single or repeated oral administration of the drug. Serum and urine samples from healthy humans after a single oral administration have also been analysed. The identity of gas–liquid chromatographic peaks has been established by mass spectrometry. In blood serum and brain, only pinazepam and its N-depropargylated product (demethyldiazepam) were found; from urine, 3-hydroxypinazepam and oxazepam were also recovered. The sensitivity of the gas–liquid chromatographic method is of the order of 5–10 ng of pinazepam and 15–20 ng of the other three benzodiazepines per ml of serum.

INTRODUCTION

Pinazepam (7-chloro-5-phenyl-1-propargyl-1,4-benzodiazepin-2-one), a compound with ansiolitic activity, has recently been synthesized in the Zambeletti research laboratories[1]. The *in vivo* kinetics and bio-transformation of pinazepam in rats and dogs were studied by means of the tritiated compound[2], and it was thus established that N-depropargylation and C-hydroxylation at position 3 were the major metabolic pathways of pinazepam (Fig. 1.).

These pathways were confirmed by using gas–liquid chromatography (GLC) coupled with mass spectrometry (MS), the four benzodiazepines being identified and determined by GLC, and the identity of the GLC peaks being confirmed by MS.

EXPERIMENTAL

Reagents and standards

The reagents used were hydrochloric acid R.S. and sodium hydroxide R.S. (Carlo Erba, Milan, Italy); diethyl ether, anhydrous acetone, *n*-hexane and *n*-heptane, pesticide grade (B.D.H., Poole, Great Britain); 1 *M* buffer solution (pH 9) of boric acid–sodium carbonate–potassium chloride; 1 *M* phosphate buffer solution (pH 5.3);

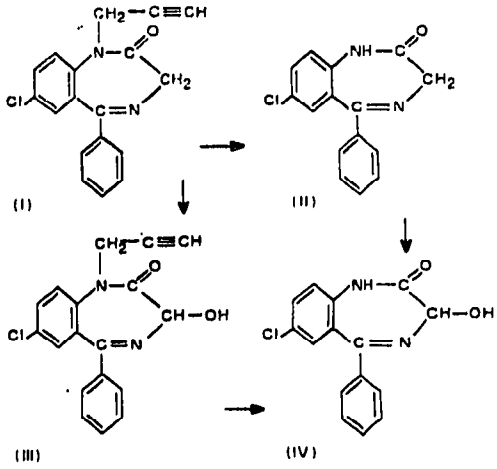

Fig. 1. Metabolism of pinazepam: I = pinazepam; II = demethyldiazepam; III = 3-hydroxypinazepam; IV = oxazepam.

and ketodase enzyme (Warner-Chilcott, Morris Plains, N.J., U.S.A.). All reagents were of analytical reagent grade (purity >99%), and all inorganic reagents were made up in doubledistilled water. The standards oxazepam[3] and demethyldiazepam[4] were suitably purified in our laboratories by crystallization. 3-Hydroxypinazepam was synthesized in our laboratories by reaction of oxazepam with propargyl bromide in dimethylformamide medium in presence of sodium hydride.

Before administration, pinazepam was "micronized" to give microcrystalline particles, 95% of which were less than 5 μm in length.

Animals

The test animals were beagle dogs (body weight 10–12 kg) and Wistar rats (body weight 200–250 g) of our own breeding; they were maintained on a standard diet and in standardised conditions and were fasted overnight before experiments.

Administration

Pinazepam, suspended in 1% carboxymethylcellulose solution, was administered to the dogs at the level of 10 mg/kg and to the rats at the level of 50 mg/kg; human volunteers received a single dose of 5 mg in a hard gelatin capsule.

Gas chromatography

Apparatus. A Perkin-Elmer Model F 30 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The glass column (90 cm × 6 mm I.D.) was packed with Gas-Chrom Q (60–80 mesh) (Applied Science Labs., State College, Pa., U.S.A.) coated with 3% of GE XE-60; it was conditioned for 1 h at 150° with a nitrogen flow-rate of 55 ml/min, then for 4 h at 240° without nitrogen and for 24 h at 250° with a nitrogen flow-rate of 55 ml/min.

Operating conditions. Column temperature, 250°; injection-port temperature, 250°; detector temperature, 300°; carrier-gas (nitrogen) flow-rate, 55 ml/min.

Preparation of standards and calibration curves

A 10-mg portion of each compound was weighed into separate 10-ml flasks and dissolved in 1 ml of absolute ethanol; each solution was then diluted to 10 ml with *n*-hexane–acetone (4:1). These solutions, containing 1 mg/ml of each compound, were so diluted as to give concentrations of 10, 1 and 0.1 μg/ml. Of these solutions, 0.3-μl portions were injected into the gas chromatograph in order to establish the calibration curves (Fig. 2) for pinazepam and its three metabolites. The minimum amounts detectable by this GLC method were 0.1 ng for pinazepam and 1 ng for each of the other benzodiazepines.

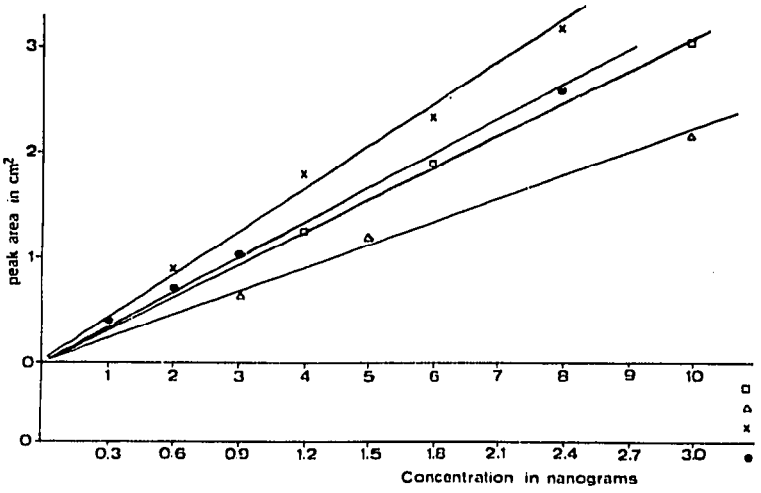

Fig. 2. Calibration curves for pinazepam (●), 3-hydroxypinazepam (△), oxazepam (×) and demethyldiazepam (□).

Mass spectrometry

The GLC–MS analysis was applied to one extract of rat brain. The mass spectra were obtained by using an LKB 9000 instrument, with a carrier-gas (helium) flow-rate of 30 ml/min and the other GLC conditions as described above. When the direct-inlet system was used, the probe temperature ranged from 160° to 190°. The following conditions were used: ionization energy 70 eV; ion-source temperature, 290°; accelerating voltage, 3.5 kV; trap current, 60 μA.

The spectra thus obtained for demethyldiazepam and oxazepam were identical with those reported previously[5]; the mass spectra of pinazepam and 3-hydroxypinazepam are shown in Fig. 3. In order to show that 3-hydroxypinazepam was not thermally decomposed during GLC–MS, an extract from urine was applied to a thin-layer chromatographic (TLC) plate and a chromatogram was developed utilizing the system described under *Thin-layer chromatography* as solvent; the spot at R_F 0.29 was scraped off and eluted with methanol, and the solution was introduced into the mass spectrometer via the direct-inlet system. The spectrum thus obtained could not be distinguished from that resulting from the GLC–MS. The R_F values of the four standards in the TLC procedure, and the retention times in GLC, are shown in Table I.

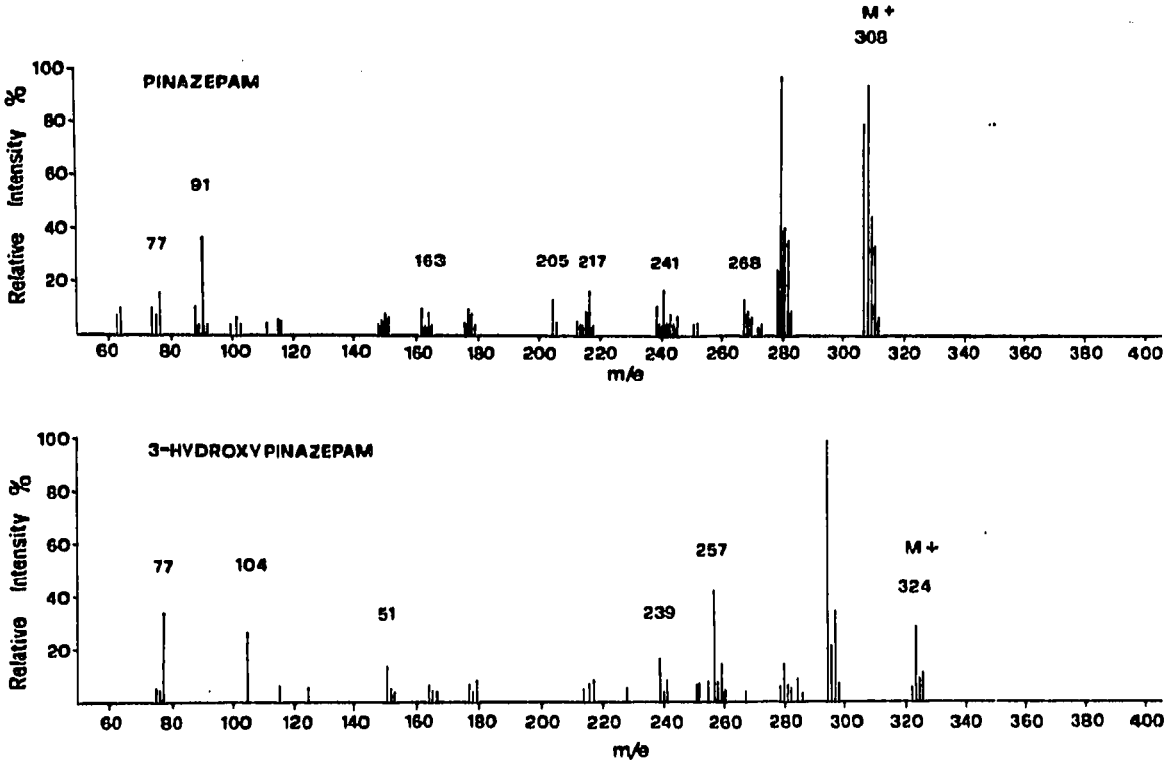

Fig. 3. Mass spectra of pinazepam and 3-hydroxypinazepam.

TABLE I

RETENTION TIMES AND R_F VALUES OF PINAZEPAM AND ITS METABOLITES

Compound	*GLC retention time (sec)*	*TLC R_F value*
Pinazepam	120	0.39
Demethyldiazepam	220	0.20
3-Hydroxypinazepam	46	0.29
Oxazepam	60	0.12

Procedure for serum extraction

A modification of the method of De Silva and Puglisi[6] was used. Six rats were sacrificed 1 h after administration of pinazepam, six rats after 2 h, and another six after 4 h; blood was withdrawn and stored separately for 1 h at 22°. With the dogs and humans, serum was sampled 1, 2 and 4 h after administration of the drug.

In a stoppered 50-ml centrifuge tube were placed 2 ml of serum and 8 ml of borate buffer solution (pH 9), then 10 ml of diethyl ether were added, and the mixture was shaken mechanically for 10 min. After separation of the organic phase, the extraction was repeated. Both ethereal solutions were combined, 5 ml of 2 *M* hydrochloric

acid were added, and the mixture was shaken for 10 min. The ethereal phase was removed, the acid phase was washed twice with diethyl ether and made alkaline by adding 5.5 ml of 2 *M* sodium hydroxide, and, after checking that the pH was 9, the mixture was extracted twice with diethyl ether, with shaking for 10 min each time.

The combined ethereal extracts were evaporated to dryness on a water-bath under vacuum at 35°, and the vacuum-dried residue was dissolved in 0.01 ml of *n*-hexane–acetone (4:1). A 0.3-μl aliquot of this solution was used for GLC. As a control, serum from untreated rats was extracted in the way described above.

In Fig. 4 are shown gas chromatograms of the four authentic standards in a solvent, and the same added to the control serum. Fig. 5 shows the chromatogram of the control serum, and Figs. 6 and 7 show those of sera from rats, dogs and humans. From these figures, it can be seen that animal serum contains only pinazepam and demethyldiazepam.

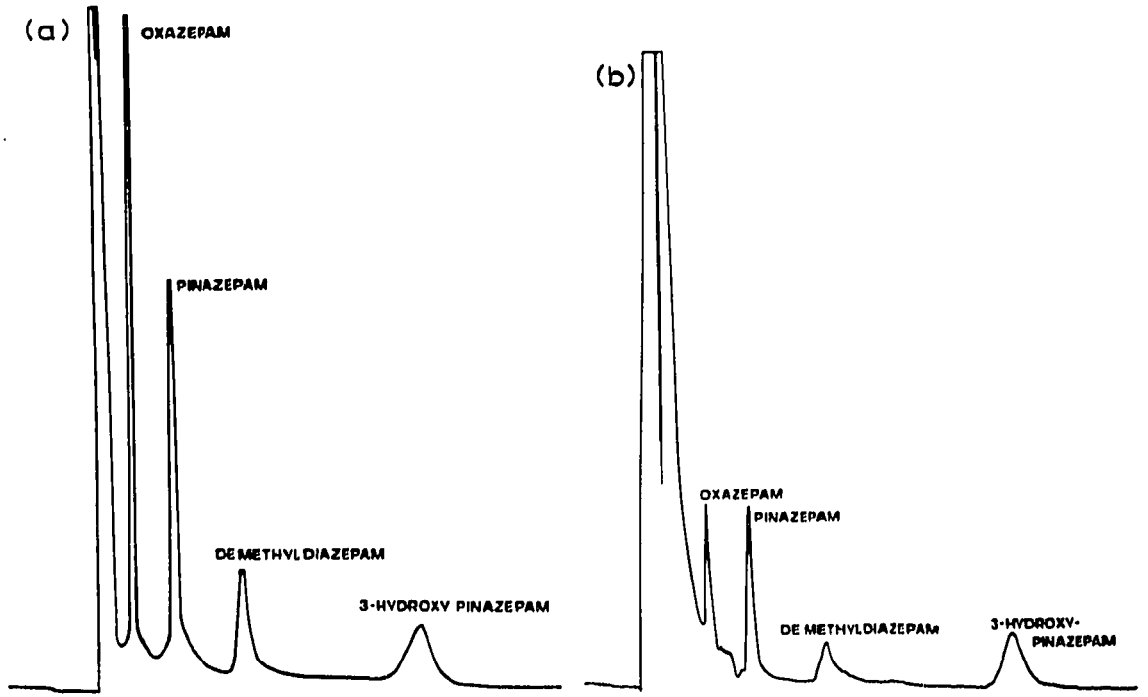

Fig. 4. Gas chromatograms of (a) the four standards (pinazepam and its metabolites), and (b) an extract of rat serum containing the four authentic standards.

Thin-layer chromatography

From the foregoing, it appeared that oxazepam and 3-hydroxypinazepam were completely absent from sera of rats, dogs and men. To ascertain the absence of these metabolites, we subjected pooled sera from five rats treated with pinazepam to all the procedures described above. The diethyl ether was carefully removed from the final solution, the residue was dissolved in *n*-hexane–acetone (4:1), and this solu-

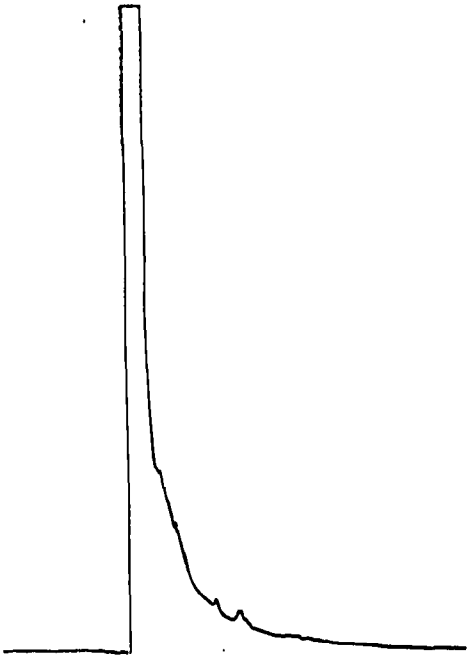

Fig. 5. Gas chromatogram of an extract of control rat serum.

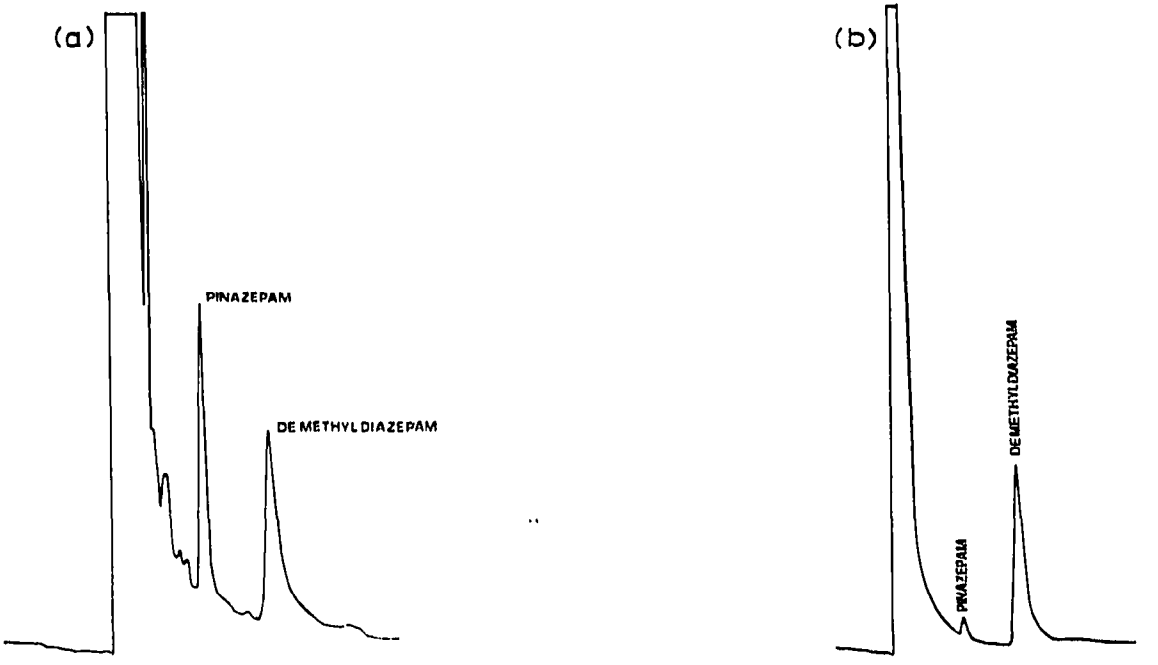

Fig. 6. Gas chromatograms of an extract of rat serum (a) and dog serum (b) after a single oral administration of pinazepam.

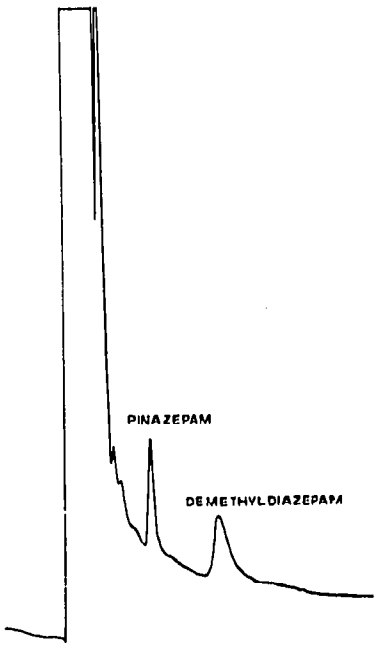

Fig. 7. Gas chromatogram of an extract of human serum after a single oral administration of pinazepam.

tion was chromatographed on a 0.25-mm layer of Merck silica gel F_{254}, with chloroform–*n*-heptane–ethanol–acetone (20:20:2:1) as developing solvent.

Pure standards of oxazepam and 3-hydroxypinazepam were chromatographed alongside the samples, and, after development, the sorbent was scraped from the zones corresponding to these two metabolites. The silica gel so obtained was extracted with diethyl ether, the solvent was evaporated *in vacuo*, and 0.3 μl of a solution of the residue in *n*-hexane–acetone (4:1) was submitted to GLC. The resulting gas chromatogram showed no peaks for oxazepam or 3-hydroxypinazepam, which suggests that these hydroxylated compounds are eliminated almost immediately in the urine.

Procedure for preparing brain extracts

Immediately after the rats had been killed, the brains were homogenized in a glass Potter–Elvehjem apparatus containing buffer solution at pH 9. The homogenate was centrifuged at 3000 *g* for 30 min in a refrigerated centrifuge, and a portion of the supernatant liquid (equivalent to 0.5 or 1 g of tissue) was extracted in the way already described for blood serum. A sample of the extract was subjected to GLC; the resulting chromatogram is shown in Fig. 8.

In this chromatogram, only peaks for pinazepam and demethyldiazepam appear. A small peak due to oxazepam appeared only after pooling six extracts, subjecting them to TLC, removing the spot at $R_F = 0.12$ (located in ultraviolet radiation), eluting it with methanol and injecting 0.3 μl of the methanolic extract into the gas chromatograph.

The amount of demethyldiazepam in brain is about four times the amount of

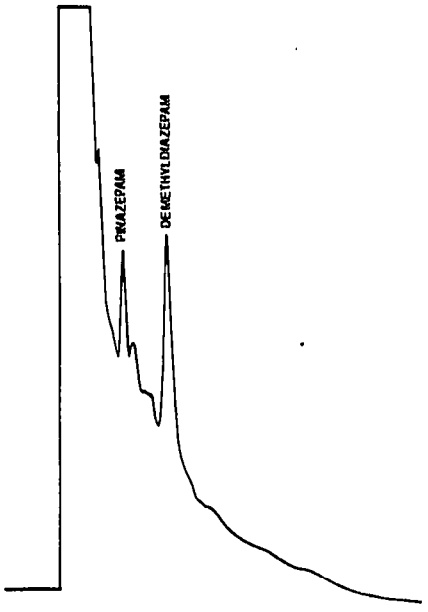

Fig. 8. Gas chromatogram of extract of rat brain.

pinazepam, whereas oxazepam accounts for about 1 % of the sum of the three benzodiazepines present.

Procedure for urine extraction

Determination of unconjugated oxazepam and 3-hydroxypinazepam. The filtered urine (5 ml) was adjusted to pH 9 and extracted as described for serum, but with 6 *M* hydrochloric acid instead the 2 *M* acid. The resulting gas chromatogram showed that pinazepam and all its metabolites were present in rat urine (Fig. 9). It is well-known[7] that hydroxy derivatives of benzodiazepines are for the most part present in urine as glucuronides; thus, the amounts of oxazepam and 3-hydroxypinazepam determined represented only the free metabolites.

Determination of total amount of oxazepam and 3-hydroxypinazepam. To 5 ml of urine were added 5 ml of phosphate buffer solution of pH 5.3 and 0.1 ml of ketodase, and the mixture was incubated at 37°, with mild agitation. The solution was then cooled, its pH was adjusted to 9, and extraction was carried out as described above for the untreated urine; 5-ml portions of untreated urine were similarly extracted. From the chromatograms of the resulting extracts, both free and conjugated oxazepam and 3-hydroxypinazepam could be determined. The results showed that 86 % of the oxazepam present in rat urine is in conjugated form; the corresponding figure for dog urine is 93 %. For 3-hydroxypinazepam, 88 % of this metabolite is present in conjugated form in rat urine and 95 % in dog urine (Table II). The values for pinazepam and its metabolites in urine from rats, dogs and humans are compared in Table III, from which it can be seen that oxazepam is always the major urinary metabolite.

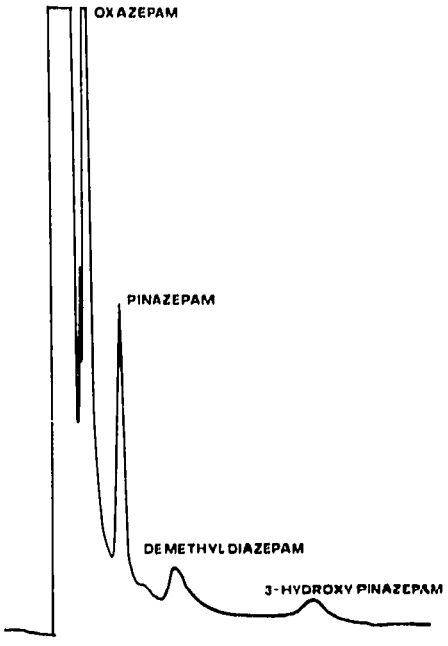

Fig. 9. Gas chromatogram of extract of rat urine.

TABLE II

ESTIMATION OF FREE AND CONJUGATED FORMS OF OXAZEPAM AND 3-HYDROXY-PINAZEPAM IN ANIMAL URINE AFTER INGESTION OF PINAZEPAM

Each result is the mean of six determinations.

Animal	*Initial dose per kg of body weight (mg)*	*Oxazepam (%)*		*3-Hydroxypinazepam (%)*	
		Free	*Conjugated*	*Free*	*Conjugated*
Rat	50	14	86	12	88
Dog	10	7	93	5	95

TABLE III

ESTIMATION OF PINAZEPAM AND ITS METABOLITES IN URINE AFTER A SINGLE ORAL DOSE OF PINAZEPAM

Each result (mean ± standard deviation) is the mean of six determinations.

Species	*Dose per kg of body weight (mg)*	*Oxazepam (%)*	*3-Hydroxypinazepam (%)*	*Pinazepam (%)*	*Demethyldiazepam (%)*
Rat	50	68.86 ± 7.34	1.10 ± 0.1	15.15 ± 2.8	14.88 ± 3.77
Dog	10	94.12 ± 3.37	1.07 ± 0.95	0.50 ± 0.30	4.43 ± 3.14
Man	5*	88.2 ± 3.03	—	3.2 ± 1.35	9.8 ± 2.34

* Total dose.

RESULTS AND DISCUSSION

The peaks for pinazepam and its metabolites were identified by their retention times in comparison with those of compounds previously synthesized, and their identities were confirmed by GLC–MS. The peak areas were determined by measuring the peak heights and widths at half-height using the sloping-baseline technique.

The method we have developed is useful for determining pinazepam and its metabolites in samples of blood serum, urine and brain from animals that have received one or more doses of the drug. Moreover, this method gives highly reproducible results. The recovery of the four substances added to serum or to urine is 84.33 % for pinazepam, 79.67 % for demethyldiazepam, 76.33 % for 3-hydroxypinazepam and 83.3 % for oxazepam (Table IV). The percentage recovery of benzodiazepin-2-ones was determined by using a direct calibration curve. Our method differs from that of De Silva and Puglisi in the GLC conditions and in the pH used for extracting the metabolites; we found that the best pH for complete extraction was 9.

TABLE IV

RECOVERIES OF PINAZEPAM AND ITS METABOLITES ADDED TO SERUM AND URINE

Each result is the mean of six determinations.

Drug	*Amount added (ng)*	*Recovery (%)*
Pinazepam	200	82
	100	86
	50	85
		Mean ± S.D. 84.33 ± 1.36
Demethyldiazepam	200	78
	100	81
	50	80
		Mean ± S.D. 79.67 ± 1.02
3-Hydroxypinazepam	200	77
	100	76
	50	76
		Mean ± S.D. 76.33 ± 0.34
Oxazepam	200	84
	100	81
	50	85
		Mean ± S.D. 83.33 ± 1.36

During the GLC of diazepam and its metabolites, oxazepam is eluted first, despite the fact that it is the most polar compound; this behaviour is attributed to thermal decomposition. In fact, oxazepam loses a molecule of water on the column at temperatures over 200°, giving 6-chloro-4-phenylquinazoline-2-carboxaldehyde; this compound is more volatile than pinazepam and its other two metabolites, which are eluted intact. Only pinazepam and demethyldiazepam are present in serum, and serum levels of these two compounds are shown in Table V. The amount of demethyldiazepam always exceeds that of pinazepam, generally by four or five times. We can

BLE V

RUM LEVELS (ng/ml) OF PINAZEPAM AND DEMETHYLDIAZEPAM AFTER A SINGLE DOSE NAZEPAM

ch result (mean ± standard error) is the mean of six determinations.

ecies	*Dose per kg of body weight (mg)*	*Time after pinazepam administration*					
		1 h		*2 h*		*4 h*	
		Pinazepam	*Demethyl-diazepam*	*Pinazepam*	*Demethyl-diazepam*	*Pinazepam*	*Demethyl-diazepam*
t	50	534 ± 67	2561 ± 190	421 ± 63	1984 ± 220	273 ± 54	1315 ± 3
g	10	8 ± 2	31 ± 7	14 ± 4	468 ± 126	15 ± 3	658 ± 14
n	0.07	14 ± 2	52 ± 13	16 ± 5	70 ± 19	17 ± 5	72 ± 1

say that, in blood, only pinazepam and demethyldiazepam are present, while in rat brain a very small amount of oxazepam is also present. If we consider Table II, we can affirm that oxazepam is the major urinary metabolite of pinazepam for all the animal species tested; this is in accord with the known metabolism of all other benzodiazepines.

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