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RAPID AND SENSITIVE ELECTRON-CAPTURE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PINAZEPAM AND ITS METABOLITES IN HUMAN PLASMA, URINE AND MILK

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SUMMARY

A rapid, sensitive and specific gas–liquid chromatographic method for the measurement of pinazepam and its metabolites in biological fluids is reported. After a single extraction of the sample with toluene, the organic phase is concentrated and, after chromatography on a 3% OV-17 column, measured with an electron-capture detector.

The sensitivity was 1.0 ng/ml for pinazepam and 5.0 ng/ml for its metabolites. Plasma levels and urinary excretion in human volunteers and plasma and milk levels in women suffering from anxiety during breastfeeding are reported.

INTRODUCTION

Pinazepam (Pz) (7-chloro-1,2-dihydro-1-propargyl-5-phenyl-3H-1,4-benzodiazepin-2-one, Fig. 1) is a new benzodiazepine derivative with anxiolytic activity. In order to study the pharmacokinetics of Pz and its metabolites, an electron-capture gas–liquid chromatographic (GLC–ECD) method has been used. A GLC–ECD assay for determining Pz and its metabolites has been published recently[1,2], but our method appears to be more rapid and sensitive without any reduction in specificity.

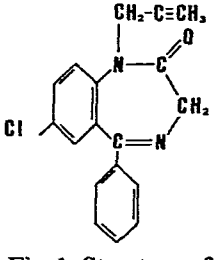

Fig. 1. Structure of the pinazepam molecule.

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Up to now most GLC methods for the analysis of benzodiazepine derivatives, including the recently reported assay for Pz, are modifications of the original methods[3,4]. These methods are very reproducible and sensitive but require three or more steps in the extraction procedure.

In our previous research, it was observed that N-demethyldiazepam (N-Dz) can be easily extracted in toluene and analyzed by means of GLC, and two GLC–ECD methods[5,6] for determining some benzodiazepine derivatives after a single extraction with an organic solvent have been developed. The advantages of these methods are their rapidity and simplicity.

In order to study the pharmacokinetics of Pz, which is an interesting benzodiazepine on account of its metabolic breakdown pattern, we have modified the GLC–ECD method so that the drugs are extracted only once with toluene. Toluene is preferred to other organic solvents because of the good recovery of the drugs, the extraction of few endogenous ECD-detectable substances and its relatively low toxicity.

EXPERIMENTAL

Materials

Pinazepam (Pz), N-demethyldiazepam (N-Dz), oxazepam (Ox) and 3-hydroxypinazepam (3-OH-Pz) were supplied by Zambeletti (Milan, Italy) and lorazepam (Lz) (used as an internal standard) by Wyeth (Rome, Italy). The following reagents were used: toluene, scintillation grade (Merck, Milan, Italy), acetone (Merck), dipotassium hydrogen orthophosphate, hydrochloric acid and sodium hydroxide (Carlo Erba, Milan, Italy), Ketodase (β-glucuronidase, 5000 u/ml) (Angiolini, Milan, Italy) and trimethylchlorosilane (Fluka, Milan, Italy). Toluene and acetone were refluxed on 1.5-m columns; 2 l of each solvent were refluxed, the first and last 100 ml being discarded and the remainder collected and kept in a dark bottle.

The standard compounds were dissolved together in acetone and dilutions made to the desired concentrations. Three standard solutions were prepared for dosing the drugs in biological specimens:

(A) For plasma and urine: Pz = 0.2 ng/μl; N-Dz = 1.0 ng/μl; Ox = 1.0 ng/μl; no 3-OH-Pz.

(B) For milk: Pz = 0.05 ng/μl; N-Dz = 0.25 ng/μl; Ox = 0.25 ng/μl; no 3-OH-Pz.

(C) For urine (conjugated drugs): Ox = 2.5 ng/μl; 3-OH-Pz = 0.5 ng/μl; N-Dz = 0.5 ng/μl; Pz = 0.5 ng/μl.

Standard 3-OH-Pz was not included in standard solutions A and B because it was not present as a free metabolite in these sources.

A Carlo Erba Fractovap G-1 gas chromatograph, equipped with a nickel-63 electron-capture detector, was used. A Pyrex glass column (2.5 m long × 4 mm I.D.) was silanized for 6 h with a 5% solution of trimethylchlorosilane in *n*-hexane, dried at 150° and packed with Gas-Chrom Q (100–120 mesh) coated with 3% OV-17 (Applied Science Labs., State College, Pa., U.S.A.). The column was then conditioned for 1 h at 250° (with a nitrogen flow of 40 ml/min), 4 h at 340° (without nitrogen) and 24 h at 275° (with a nitrogen flow of 40 ml/min). The operating conditions were: injection port temperature, 275°; column temperature, 264°; detector temperature, 270°; carrier and scavenger gas (high-purity nitrogen) flow-rates, 60 and 20 ml/min, respec-

tively. The attenuation input was 100 and the output generally 64. The detector pulse space was 30 μsec, pulse width 10 μsec and pulse amplitude 20 V. The recorder was a Leeds & Northrup Italiana (Milan, Italy) Speedomax XL 681, with a chart speed of 1 cm/min.

Extraction procedures

Various amounts of standard solution A and 50 μl of Lz (0.1 ng/μl) in acetone as internal standard were placed in 10-ml stoppered test-tubes, then 1 ml of 1 *M* dipotassium hydrogen orthophosphate buffer (pH 7.5), 0.5 ml of beef plasma (to simulate human plasma) and 5 ml of toluene were added with an automatic pipette. The test-tubes were shaken for 20 min and, after centrifugation at 1100 *g* for 10 min, 4 ml of the organic phase were transferred into conical test-tubes and dried at 45° under a gentle stream of high-purity nitrogen. The residues were dissolved in 50 μl of acetone and 1–2 μl was injected for GLC analysis.

The extraction procedure from milk was performed as described above, 1 ml of milk (from a commercial source, to simulate human milk) being assayed. Standard solution B was used for the calibration graph.

For unconjugated drugs in the urine, 2 ml of urine were added to 1 ml of 1 *M* dipotassium hydrogen orthophosphate buffer (pH 7.5); the pH of the urine–buffer mixture was 7.4–7.3. No differences were observed in the recovery of drugs compared with Arnold's method[5]. The extraction was performed as described above.

Dosage of subjects and collection of samples

Firstly, three healthy volunteers, free from drugs for at least 4 weeks, received 10 mg of Pz at 8 a.m. in a single oral dose and had a light breakfast about 90 min later. Blood was withdrawn at various times after the ingestion of Pz, centrifuged at 4° and the plasma was immediately frozen and stored at −20° until required for analysis. Urines were collected at various times after drug administration, the volume and pH accurately measured, and a frozen aliquot was stored at −20° until required for analysis.

Secondly, four pregnant women suffering from anxiety received Pz (10 mg *per os*) 2–3 h before the delivery. Blood and milk were drawn once a day, immediately before suckling, during the 4 days after the delivery. The milk and plasma were immediately frozen and stored at −20° until required for analysis.

RESULTS

Fig. 2 shows a chromatogram of the standard compounds and of the toluene extracts from plasma, urine and milk. The retention time of the parent drug, metabolites and internal standard are listed in Table I. The calibration graphs for drugs were obtained by plotting the ratio of the peak area to that of an internal standard against known amounts of the drugs added to the biological fluids. The peak area was calculated by multiplying the peak height by the width at half-height.

The recovery is illustrated by Fig. 3, which shows an external and internal calibration graph for each compound after a single extraction from plasma with 5 ml of toluene. The recoveries were 77.50 ± 0.64% for Pz, 95.00 ± 1.35% for N-Dz, 93.37 ± 0.90% for Ox and 90.03 ± 0.82% for 3-OH-Pz. No differences were found

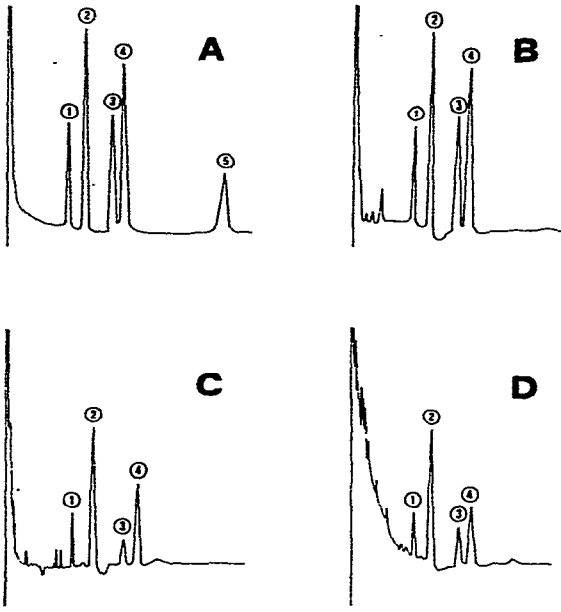

Fig. 2. Typical chromatograms obtained after injection of 1 μl of a solution of standard compounds (A), an extract from plasma (B), milk (C) and urine (unconjugated drugs) (D). Peaks: 1 = oxazepam; 2 = lorazepam (internal standard); 3 = pinazepam; 4 = demethyldiazepam; 5 = 3-hydroxy-pinazepam.

TABLE I

RETENTION TIMES OF THE DRUGS TESTED

For the GLC operating conditions, see Experimental.

Drugs	*Retention time*
Oxazepam	3 min 18 sec
Lorazepam	4 min 12 sec
Pinazepam	5 min 45 sec
N-Demethyldiazepam	6 min 18 sec
3-Hydroxypinazepam	11 min 25 sec

between the recoveries from milk and urine. Lz, used as the internal standard, was extracted with a high recovery and interfering peaks were not detected. The minimal amounts of the drugs that could be detected were 1.0 ng/ml for Pz and 5 ng/ml for the other compounds.

In order to test the reproducibility of the method, 50 μl of acetone solution containing 0.1 ng/μl of Pz, 0.5 ng/μl of N-Dz, 0.5 ng/μl of Ox and 0.5 ng/μl of 3-OH-Pz were placed in seven test-tubes. The internal standard Lz, 0.5 ml of plasma and 1.5 ml of buffer were added. These samples were analyzed in parallel with a calibration curve. The results are shown in Table II.

As a preliminary test of the method, trials were carried out with human subjects. Fig. 4 shows the plasma levels of Pz and N-Dz at different times after a single administration of Pz (10 mg *per os*) to three healthy volunteers. On each occasion N-Dz levels were higher than those of Pz, and started to decrease slightly only after

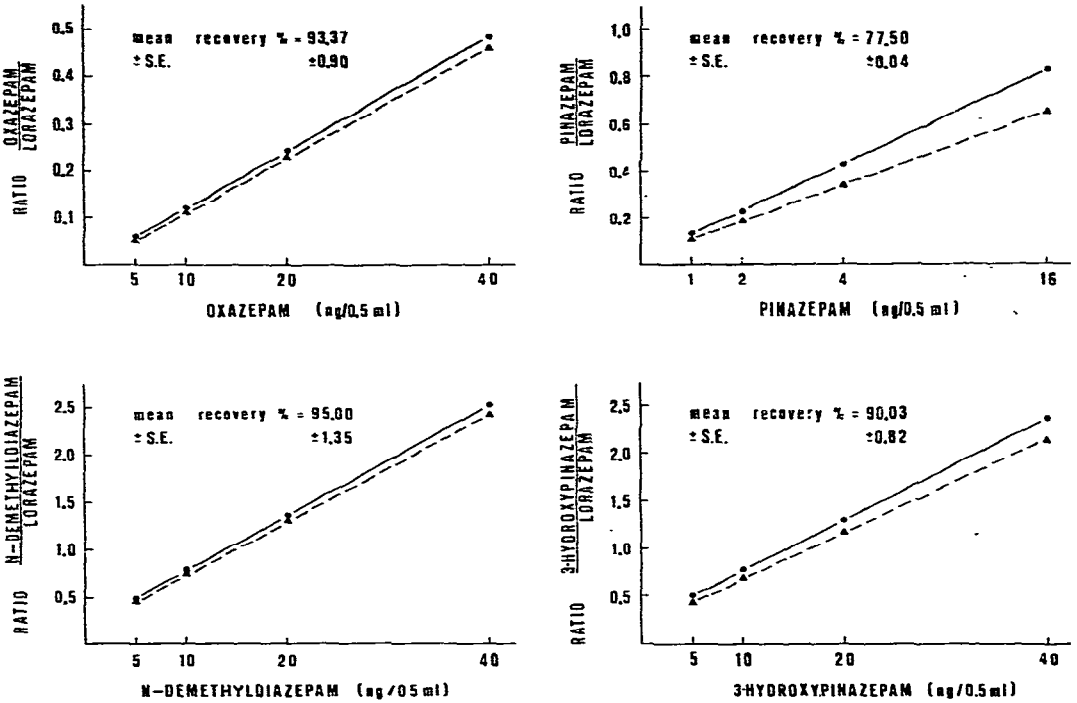

Fig. 3. Calibration graphs for the drugs studied. The continuous lines are the external standard graphs and the broken lines the internal standard graphs. The latter were obtained after extraction of 0.5 ml of plasma + 1.5 ml of phosphate buffer (1 *M*; pH 7.5).

TABLE II

REPRODUCIBILITY OF THE METHOD

Each value for "Amount found" represents the determination of a known amount of drug added to plasma, and is the result of a single GLC analysis. The reproducibility was tested on seven different samples each containing the four drugs.

Amount found	*Drug and amount added (ng)*			
	Pinazepam (5.0)	*N-Demethyl-diazepam (25.0)*	*Oxazepam (25.0)*	*3-Hydroxy-pinazepam (25.0)*
Amount found for 7 different samples (ng)	5.0	25.0	26.0	26.0
	4.8	24.6	24.5	25.2
	5.4	26.0	27.5	27.0
	5.2	25.0	25.3	25.4
	4.8	26.5	26.5	26.2
	5.3	25.2	25.1	25.6
	4.8	25.0	25.0	25.0
Mean ± standard error (ng)	5.04 ± 0.10	25.33 ± 0.25	25.70 ± 0.39	25.77 ± 0.26
Standard deviation (ng)	0.26	0.67	1.03	0.69

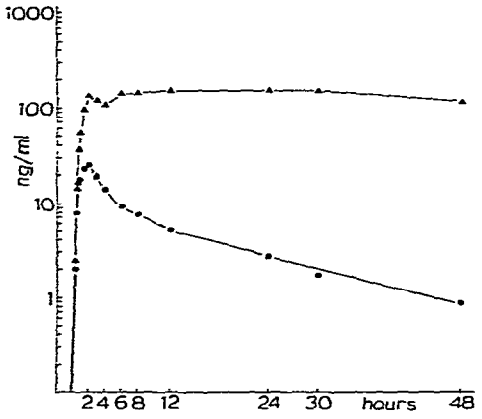

Fig. 4. Plasma levels (ng/ml) of pinazepam (●) and N-demethyldiazepam (▲) in three healthy volunteers after receiving 10 mg of pinazepam in a single oral dose.

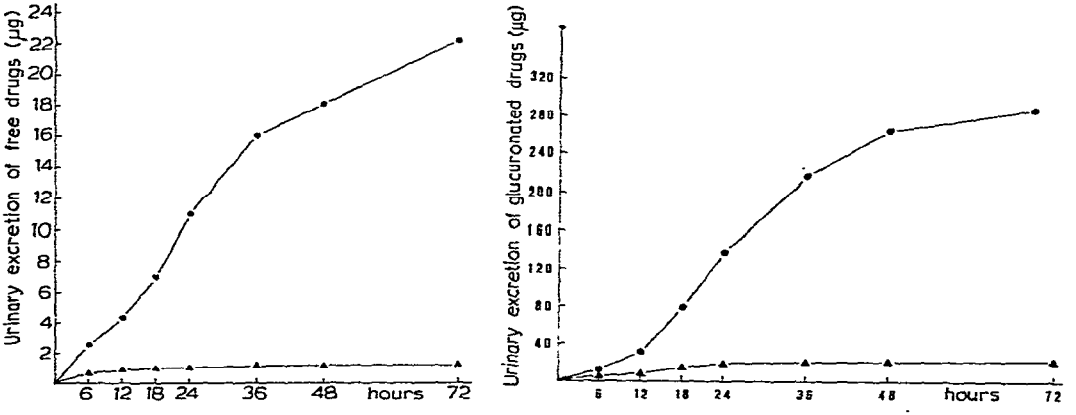

Fig. 5. Cumulative urinary excretion of free drugs at different times after a single administration of pinazepam (10 mg *per os*) in three healthy volunteers. ●, N-Demethyldiazepam; ▲, pinazepam.

Fig. 6. Cumulative urinary excretion of glucuronated drugs at different times after a single administeration of pinazepam (10 mg *per os*). ●, Oxazepam; ▲, 3-hydroxypinazepam.

TABLE III

N-DEMETHYLDIAZEPAM LEVELS IN HUMAN MILK (ng/ml) DURING THE FIRST FOUR DAYS AFTER DELIVERY

The values in parentheses are the corresponding N-demethyldiazepam plasma levels (ng/ml). Pinazepam (10 mg *per os*) was administered 2–3 h before delivery.

Subject	*Days after delivery*			
	1	*2*	*3*	*4*
P.G.	— (40)	6.2 (84)	11.2 (37.5)	—
F.E.	— (31)	6.0 (44)	5.2	—
N.G.	— (30)	— (27)	— (25)	6.2
S.M.	— (32.5)	5.0	5.0	—

30 h. In the plasma, Ox and 3-OH-Pz were not detectable. In Figs. 5 and 6 the cumulative excretion of unconjugated drugs is reported. In pregnant women suffering from anxiety, Pz (10 mg *per os*) was administered in a single dose 2–3 h before the delivery. Only N-Dz was measurable in milk (Table III).

DISCUSSION

The simplicity and speed of the analysis are the most important features of this GLC–ECD method for the determination of Pz and its metabolites. Forty samples (including those used for the calibration graph) could normally be extracted and injected for GLC analysis within 8 h. This aspect should not be underestimated for clinical pharmacological investigations and for the monitoring of drug plasma levels in hospitalized patients, where a large number of samples must be analyzed. Further, the method appears to be suitable for the GLC analysis of Lz: investigations of this possibility are in progress. In addition, the use of a structurally related internal standard (usually neglected in the traditional GLC assay for benzodiazepines) eliminates a possible source of errors in the analysis. Other advantages of the method are the reproducibility, sensitivity and linearity. On the other hand, the chromatogram is not free from impurity peaks as in the traditional methods, but no interfering peaks from endogenous substrates were noted.

Using this GLC assay, we have studied the pharmacokinetics of Pz and its metabolites in healthy volunteers and the transplacental passage of the same drugs in pregnant women suffering from anxiety. Only preliminary data are reported. Pz is rapidly absorbed by the gastrointestinal tract and quickly metabolized to N-Dz, Ox and 3-OH-Pz[2]. The investigation with healthy volunteers showed that plasma levels of N-Dz were higher than those of Pz at the times studied. Pz decreased more rapidly, while the N-Dz remained in the therapeutic range for 48 h. Ox and 3-OH-Pz were not detected in plasma, but were found as glucuronides in the urine: conjugated Ox is excreted in very large amounts.

A study conducted on pregnant women showed that the Pz and N-Dz quickly cross the placenta (data not reported), with accumulation of the latter drug in the cord plasma. After birth, N-Dz is detected in milk at levels of 5–11.2 ng/ml. Pz was present but at levels too low to measure.

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REFERENCES

1 F. Scrollini, S. Caliari, A. Romano and P. Torchio, *Arzneim.-Forsch.*, 25 (1975) 934.
2 A. Trebbi, G. B. Gervasi and V. Comi, *J. Chromatogr.*, 110 (1975) 309.
3 J. A. F. De Silva, M. A. Schwartz, V. Stefanovic, J. Kaplan and L. D'Arconte, *Anal. Chem.*, 63 (1964) 2099.
4 S. Garattini, F. Marcucci and E. Mussini, in R. Porter (Editor), *Ciba Foundation Symposium on Gas Chromatography in Biology and Medicine*, Churchill, London, 1st ed., 1969, p. 161.
5 E. Arnold, *Acta Pharmacol. Toxicol.*, 36 (1975) 335.
6 M. Gerna and P. L. Morselli, *J. Chromatogr.*, 116 (1976) 445.