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Note

Gas chromatographic determination of chlorodesmethyldiazepam and lorazepam in rats and mice

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2′-Chlorodesmethyldiazepam (I) is a new benzodiazepine. Previous studies in our laboratories[1–3] have indicated that several benzodiazepines undergo a process of N-demethylation and/or C_3-hydroxylation. C_3-Hydroxylation is a metabolic pathway common to the whole benzodiazepine class and its intensity varies according to animal species[1], age[4] and induction by other treatments[5].

Rat and mouse liver microsomal enzymes metabolized I to lorazepam (II) through C_3-hydroxylation. Lorazepam is pharmacologically active[6] in most of the tests utilized to screen benzodiazepines[3].

This study was carried out in order to establish the pharmacokinetic profile of I and its metabolite in rats and mice. This paper describes an improved, sensitive and rapid gas–liquid chromatographic (GLC) assay method for the measurement of I and its metabolite in biological tissues at the nanogram level.

EXPERIMENTAL

Chemicals

2′-Chlorodesmethyldiazepam and lorazepam of pharmaceutical-grade purity (99%) were kindly supplied by Ravizza (Muggiò, Milan, Italy) and pinazepam by Zambeletti (Baranzate, Milan, Italy).

Animals

Male Charles River rats (body weight 200 ± 10 g) and male CD_1 mice (body weight 22 ± 3 g) were used in all experiments.

GLC Determination

A Carlo Erba Fractovap Model G_1 gas chromatograph equipped with a nickel-63 electron-capture detector and a 2 m × 4 mm I.D. silanized glass column packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) was used. The carrier gas (nitrogen) flow-rate was 35 ml/min and the detector purge gas flow-rate was 35 ml/min. The temperature settings were oven 298°, injection port 305° and detector 315°.

GLC–mass spectrometry

An LKB Model 9000 mass spectrometer combined with a gas chromatograph

was used. The mass spectrometric conditions were: ionization beam, 70 eV; ion source temperature, 290°; accelerating voltage, 3.5 kV; and trap current, 60 μA. A 2 m glass column of 3% OV-17 on Gas-Chrom Q (100–120 mesh) was used, operated at 298° with a helium flow-rate of 35 ml/min.

Quantitative determination of I and its metabolite II

Proportional amounts of the two compounds dissolved in an acetone solution of the internal standard (pinazepam, 0.5 ng/μl) were injected into the GLC column. Fig. 1 shows a good linearity from 0.1 to 0.8 ng/μl for both compounds.

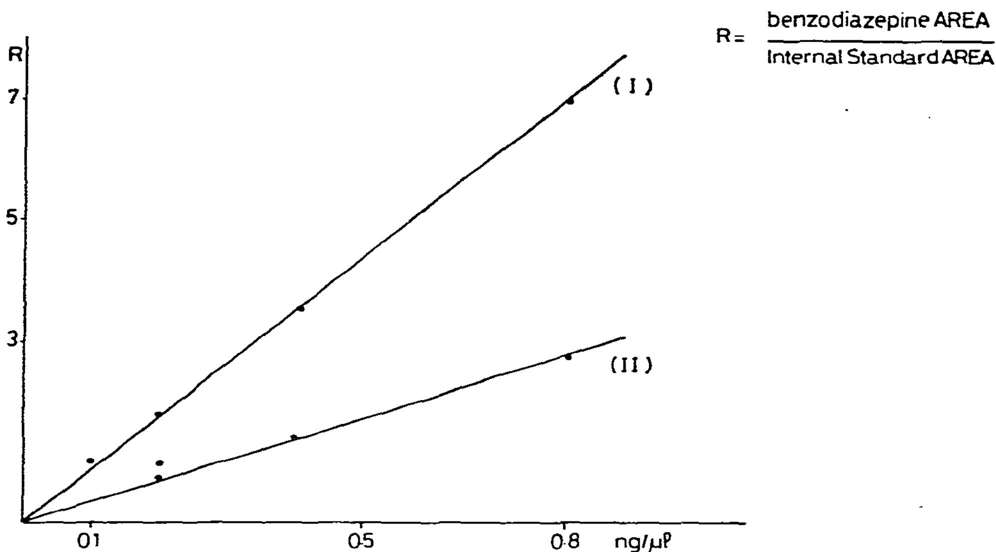

Fig. 1. Calibration graph.

Fig. 2. shows a GLC trace of I and its metabolite with the internal standard. During GLC analysis, II loses one molecule of water, as previously reported[7,8]. This pyrolysis phenomenon is common to C_3-hydroxylated benzodiazepines.

The identity of the peaks was confirmed by GLC coupled with mass spectrometry. The mass spectrum of I is reported in Figs. 3 and 4. The mass spectrum of II has been reported previously[7,8].

Drug administration

I was injected intravenously in a single dose of 5 mg/kg dissolved in a mixture of 5 mg/kg dissolved in a mixture of propylene glycol, glycofurol, benzyl alcohol and distilled water (30:30:2:48) Control animals were injected intravenously with the same volume of vehicle mixture. Groups of five animals were killed at several intervals after drug administration. Blood samples (2 ml) were collected in glass tubes containing two drops of heparin sodium in saline (2%). Immediately after death, rat and mouse brain and epididymal tissue were removed and frozen.

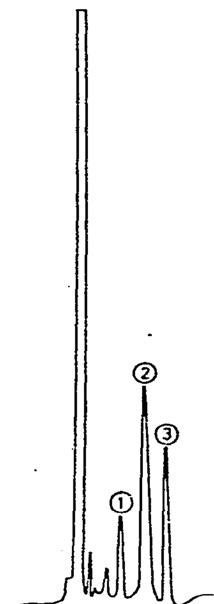

Fig. 2. GLC trace of separation between lorazepam (1) chlorodesmethyldiazepam (3) and internal standard (2).

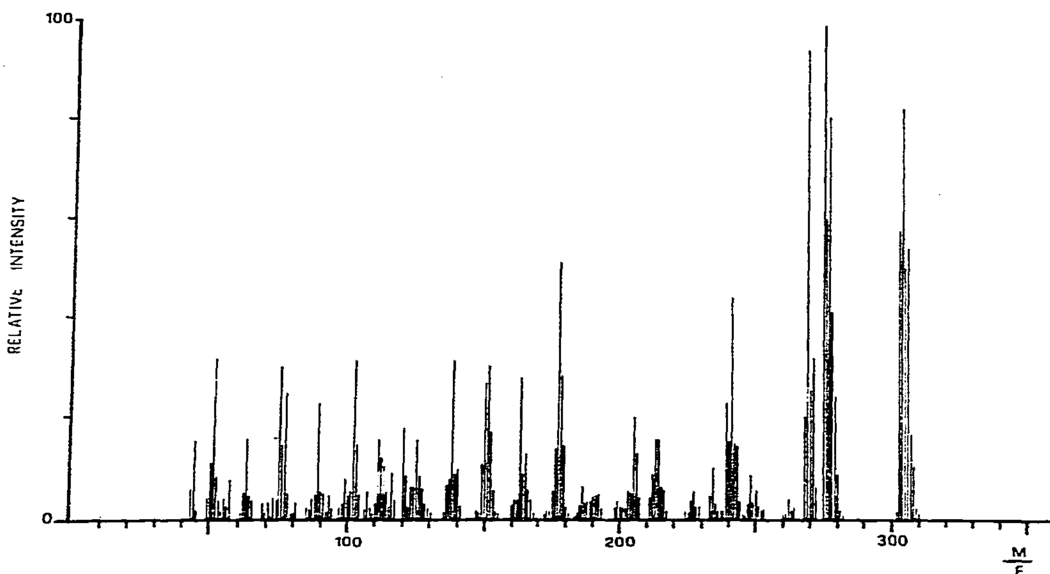

Fig. 3. Mass spectrum of chlorodesmethyldiazepam made obtained with the direct inlet system.

Extraction procedure

The extraction procedure for I and its metabolite was the same for both animal species.

To 1 ml of heparinized blood, aliquots of 50, 100 and 500 ng of I and II were

added. After the addition of 2 ml of phosphate buffer (0.1 *M*, pH 7.4), samples were extracted with 5 ml of benzene, shaken for 10 min and centrifuged at 2000 *g* for 10 min. The organic phase was collected and evaporated to dryness under vacuum. The residue was dissolved in an acetone solution of the internal standard (pinazepam, 0.5 ng/μl) and analysed by GLC.

Recoveries of I and II added ro rat and mouse blood were 93 $\pm$ 2% and 88 $\pm$ 2%, respectively.

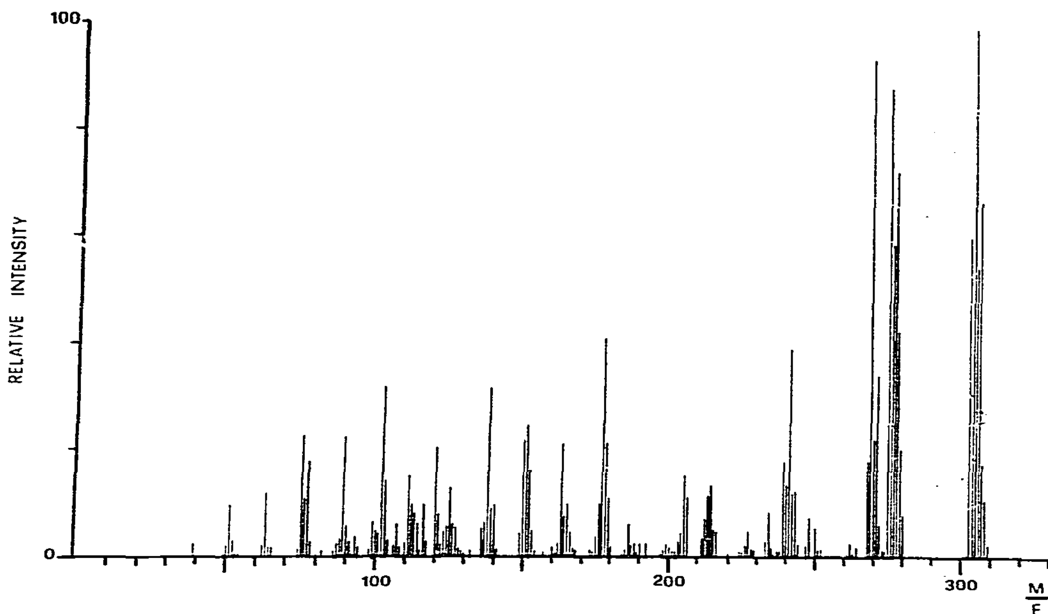

Fig. 4. Mass spectrum of chlorodesmethyldiazepam in GLC.

The extraction from rat and mouse brain and epididymal adipose tissue is more complex than that from blood as the biological samples must undergo preliminary deproteinization. Ethanol proved to be the best deproteinizing agent for subsequent GLC analysis. The samples were homogenized in ice-cold absolute ethanol (2:4, w/v). The homogenates were centrifuged at 4000 *g* for 20 min and 50 ng of I and 50 ng of II were added to the upper phase.

The samples were dried under vacuum and dissolved in 3 ml of phosphate buffer (0.1 *M*, pH 7.4), then 4 ml of benzene were added. The procedure from this point was the same as for blood extraction.

Drug recoveries of 92 $\pm$ 2% and 87 $\pm$ 2% were obtained from brain and 93 $\pm$ 2% and 86 $\pm$ 2% from epididymal tissue for the rat and mouse, respectively.

RESULTS AND DISCUSSION

Rats

The levels of I and its metabolite in rat blood, brain and adipose tissue after intravenous injection of 5 mg/kg are reported in Table I. In rat blood and brain I reaches a peak 1 min after administration, then the concentration decreases until 10 h

TABLE I

CHLORODESMETHYLDIAZEPAM (I) AND LORAZEPAM (II) LEVELS AFTER i.v. ADMINISTRATION OF 5 mg/kg OF I TO RATS

Each value is the mean of 5 determinations.

Time after administration (min)	*Blood (ng/ml ± S.E.)*		*Brain (ng/g ± S.E.)*		*Adipose tissue (ng/g ± S.E.)*	
	I	*II*	*I*	*II*	*I*	*II*
1	4214 ± 260	26 ± 2	16456 ± 2192	93 ± 5	1007 ± 90	<10
5	3271 ± 253	154 ± 2	12644 ± 722	130 ± 10	2015 ± 156	<10
30	759 ± 43	293 ± 9	5824 ± 415	279 ± 28	6036 ± 241	367 ± 26
60	373 ± 35	219 ± 15	3029 ± 255	269 ± 54	4456 ± 290	241 ± 27
180	29 ± 4	60 ± 1	70 ± 9	49 ± 5	161 ± 14	55 ± 5
600	4 ± 0.4	<10	<0.5	<10	18 ± 5	<10

after treatment. Brain levels are four times higher than in blood 1 and 5 min after drug administration. The ratio between brain and blood levels reaches 7–8 at 30 and 60 min after drug administration.

In rat epididymal adipose tissue the levels of the compound are lower than in blood 5 min after administration and reach a peak at 30 min, after which they decrease to 18 ng/g at 10 h after drug administration.

The metabolite II can be detected in rat blood and brain 1 min after drug administration. It reaches the highest concentration at 30 min, after which it decreases to below the sensitivity of the method at 10 h after treatment.

In adipose tissue II is not detectable 5 min after administration; it reaches its peak at 30 min, then decreases until it is undetectable at 10 h after treatment.

Mice

The levels of I and its metabolite II after an intravenous injection of 5 mg/kg in mice are reported in Table II. Peak blood and brain levels are present at the first assay times, decreasing gradually thereafter to 1 ng/ml at 24 h.

TABLE II

CHLORODESMETHYLDIAZEPAM (I) AND LORAZEPAM (II) LEVELS AFTER i.v. ADMINISTRATION OF 5 mg/kg OF I TO MICE

Each value is the mean of 5 determinations.

Time after administration (min)	*Blood (ng/ml ± S.E.)*		*Brain (ng/g ± S.E.)*		*Adipose tissue (ng/g ± S.E.)*	
	I	*II*	*I*	*II*	*I*	*II*
1	4498 ± 190	<10	8623 ± 560	10	885 ± 46	<10
5	3006 ± 305	<10	8681 ± 864	10	3871 ± 587	<10
30	2292 ± 75	123 ± 17	3742 ± 276	238 ± 30	4467 ± 357	163 ± 9
60	1314 ± 48	152 ± 8	3161 ± 167	264 ± 13	5037 ± 414	251 ± 23
180	1079 ± 64	184 ± 12	1398 ± 88	406 ± 9	1960 ± 106	493 ± 47
300	276 ± 8	181 ± 15	891 ± 41	297 ± 23	924 ± 55	198 ± 21
600	2 ± 0.3	75 ± 8	11 ± 0.3	133 ± 16	57 ± 3	107 ± 8
1440	1 ± 0.2	22 ± –	1 ± 0.1	49 ± 3	6 ± 0.6	<10

In adipose tissue the concentration of I rises from 1 to 60 min, when the peak is reached; 24 h after treatment a concentration of 6 ng/g was measured.

II appears in the brain, blood and adipose tissue of mice 30 min after administration of I. High levels of II are detectable in the blood and the brain between 3 and 5 h after drug treatment and between 1 and 3 h in the adipose tissue. Measurable level of II are seen in the blood and brain 24 h after administration, whereas in the adipose tissue concentrations of II are below the analytical sensitivity.

These results indicate that in mice brain II reaches the concentration required for anticonvulsant activity[9] 30 min after drug treatment. This pharmacological activity lasts for 24 h (from 30 min to 24 h the levels of II are higher than 20 ng/g).

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