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Note

Gas–liquid chromatographic determination of clobazam and N-desmethyl-clobazam in plasma

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Clobazam is a newly developed benzodiazepine with the nitrogen atoms of the heterocyclic ring in the 1,5- instead of the 1,4-position as in the best known benzodiazepines. The pharmacological properties [1—3] and clinical effects [4—6] have been reported and the compound is currently used as an anxiolytic agent.

Metabolic studies after administration of ^{14}C-labeled clobazam to man, rat, monkey and dog [7] show that the compound was rapidly and extensively metabolized. Although the structure of several metabolites has not yet been identified, one important pathway noted in all the species examined was N-desmethylation, with the formation of N-desmethyl-clobazam. As a basis for future examination of the possible pharmacological significance of N-desmethyl-clobazam, since the N-desmethyl metabolites of other compounds of the benzodiazepine series have been seen to be active [8—12], we have developed a gas—liquid chromatographic (GLC) method for simultaneous determination of clobazam and N-desmethyl-clobazam in plasma samples. The procedure is specific and sensitive; plasma level curves of both compounds after administration of clobazam to guinea pigs, have been plotted.

MATERIALS AND METHODS

Standard and reagents

Clobazam and N-desmethyl-clobazam were obtained from Hoechst (Frankfurt, G.F.R.). Diazepam (kindly supplied by Ravizza, Muggio, Italy) was used as an internal marker. Other reagents were acetone (Carlo Erba, Milan, Italy) and benzene (Pestanal grade, Hoechst).

Apparatus

A Carlo Erba Fractovap 2150 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The chromatographic column was a glass tube (1 m × 4 mm I.D.) packed with 80—100 mesh Chromosorb G AW DMCS with 5% OV-25 (Supelco, Bellefonte, Pa., U.S.A.) as the liquid phase. The column temperature was 290°, the detector temperature 300° and injector port temperature 320°. The carrier gas was nitrogen at a flow-rate of 60 ml/min.

For mass spectrometry (MS) a mass spectrometer combined with a gas chromatograph (LKB 9000) was used under the following conditions: energy of the ionization beam 70 eV; ion source temperature 250°, accelerating voltage 3.5 kV and trap current 100 μA. The gas chromatograph was operated under the same conditions as above.

Standard external calibration curves

Clobazam and N-desmethyl-clobazam were dissolved in acetone (1 μg/ml) and combined aliquots of the compounds were evaporated to dryness. The dry residues were dissolved in 100 μl of acetone containing diazepam (0.25 μg/μl) as a marker, and 1—2 μl were injected into the gas chromatographic column. The ratio of the peak areas of the compounds to that of the internal marker were linear in the range from 0.1 to 2 μg per injection.

Extraction procedure

To 0.1—0.5 ml of heparin-treated plasma 2.4—2 ml of 0.5 *M* phosphate buffer (pH 9.5) were added and the samples were extracted twice for 15 min with 5 ml of benzene. After centrifugation the benzene extracts were combined and evaporated to dryness in vacuo. The dry residue was dissolved in 100–500 μl of acetone containing diazepam (0.25 μg/μl) and 1—2 μl were injected into the gas chromatographic column.

Drug-free plasma samples with known amounts of clobazam and N-desmethyl-clobazam (10—200 ng) were analyzed concurrently with each set of unknown samples. Concentrations of both compounds in the unknown samples were obtained from the ratio of the peak areas obtained to the internal standard curves. In these experimental conditions the minimum detectable amounts were 0.1 ng per injection.

Animal studies

Male albino guinea pigs (Pelizzari, Cremona, Italy) weighing about 250 g were injected intraperitoneally (i.p.) with clobazam (10 mg/kg) and killed at various times after drug administration.

Blood samples were collected in heparinized tubes, centrifuged, and plasma was analyzed as described above.

RESULTS AND DISCUSSION

The procedure described permits rapid and specific determination of clobazam and N-desmethyl-clobazam in plasma. Extraction with benzene results in a clear extract which can be injected directly into the gas chromatographic

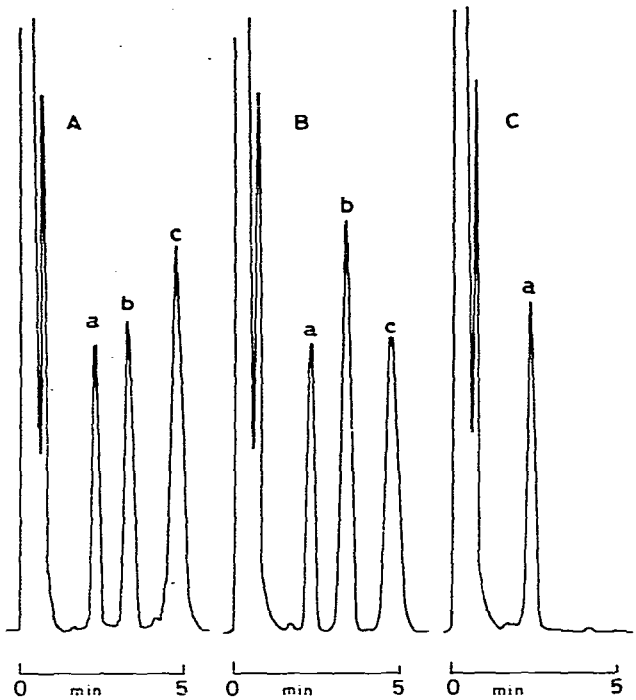

Fig. 1. Gas chromatograms of plasma extracts from guinea pigs injected with clobazam (A), from plasma to which 100 ng of both compounds were added (B) and from drug-free plasma (C). Peaks: (a) diazepam; (b) clobazam; (c) N-desmethyl-clobazam.

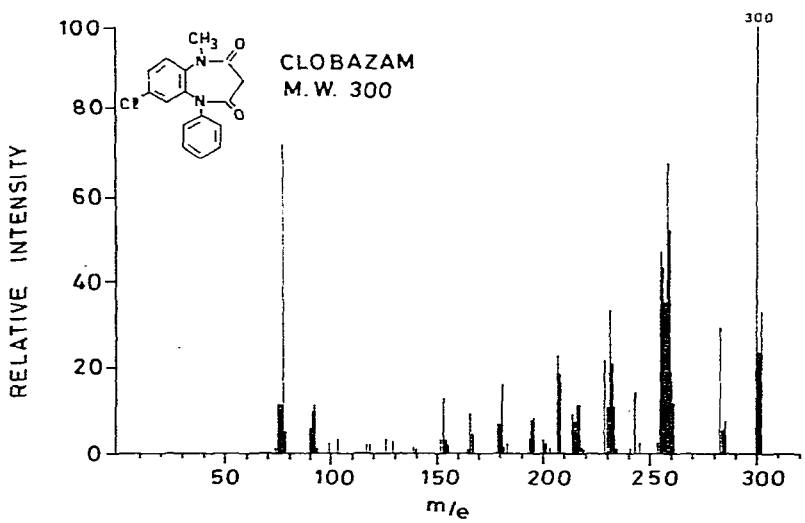

Fig. 2. Mass spectrum of clobazam.

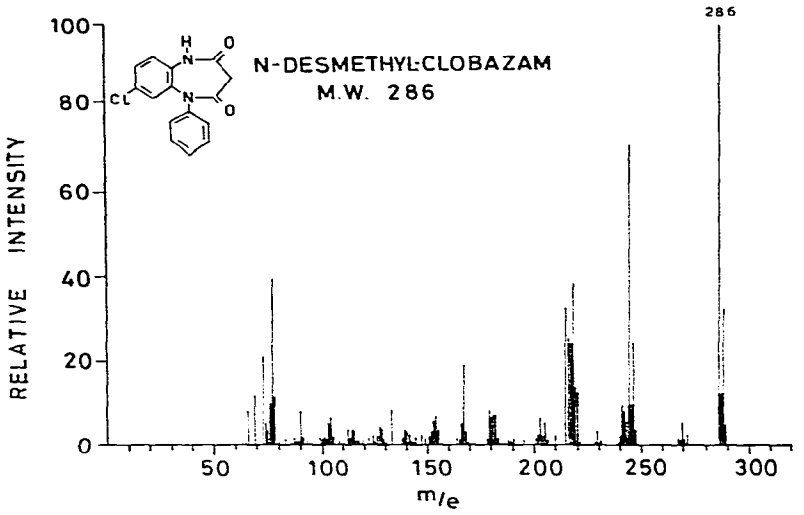

Fig. 3. Mass spectrum of N-desmethyl-clobazam.

column without further purification. In preliminary studies to choose the most suitable stationary phase for the determination of clobazam and its metabolite, 5% OV-25 on Chromosorb G AW DMCS gave the best results.

Fig. 1 presents typical gas chromatograms of extracts from plasma of guinea pigs treated with clobazam (10 mg/kg, i.p.), from plasma to which 100 ng of both compounds were added and from drug-free plasma. Retention times were 3.3 min for clobazam and 4.7 min for N-desmethyl-clobazam. Specificity of the analysis was confirmed when unknown plasma samples from guinea pigs given clobazam (10 mg/kg, i.p.) were analyzed by combined GLC—MS. The mass spectra obtained from the analysis of the GLC peaks (Figs. 2 and 3) were identical to those after injection of authentic compounds. A summary of the recovery results during kinetics studies in guinea pigs is presented in Table I. Clobazam is extracted reproducibly over the range of 10—200 ng,

TABLE I

RECOVERY OF CLOBAZAM AND N-DESMETHYL-CLOBAZAM FROM PLASMA

Each value is the mean of eight determinations.

Amount added (ng)	Clobazam		N-Desmethyl-clobazam	
	Amount found (ng ± S.D.)	Recovery (% ± S.D.)	Amount found (ng ± S.D.)	Recovery (% ± S.D.)
10	9.4 ± 0.6	94 ± 0.6	9.0 ± 0.7	90 ± 7.5
25	24.0 ± 1.4	95 ± 5.6	23.0 ± 2.2	92 ± 9.0
50	47.1 ± 2.0	94 ± 4.0	45.8 ± 2.8	92 ± 5.7
100	97.8 ± 4.4	98 ± 4.4	91.0 ± 4.4	92 ± 4.4
200	195.4 ± 4.7	98 ± 5.6	187.5 ± 11.5	94 ± 8.8

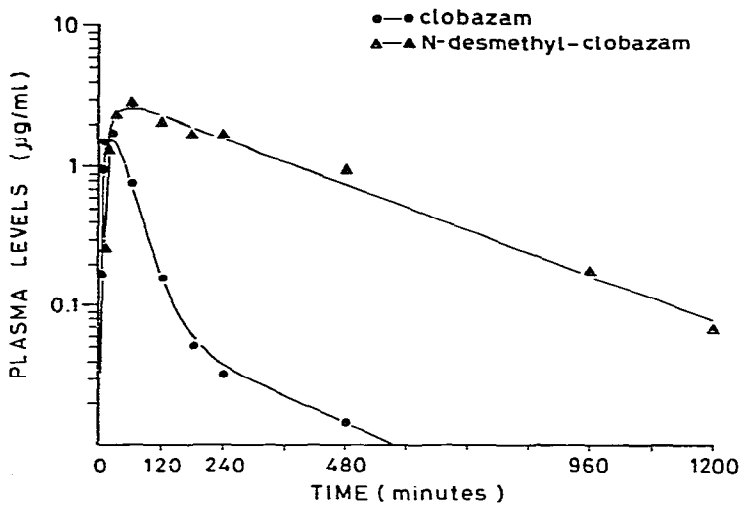

Fig. 4. Plasma level—time curves of clobazam and N-desmethyl-clobazam after intraperitoneal injection of clobazam (10 mg/kg) to guinea pigs. Each point is the mean value for six animals.

with recovery between 94–98% with a coefficient of variation (C.V.) between 4.26–6.32%. N-Desmethyl-clobazam (10–200 ng) was extracted reproducibly between 90—94% with a C.V. of 4.8–9.8%.

The validity of the analytical procedure was demonstrated by studying the plasma curves of both compounds in guinea pigs (Fig. 4) given clobazam (10 mg/kg, i.p.). Clobazam was rapidly adsorbed, rising to plasma peak concentrations between 15 and 30 min.

From the peak the plasma concentrations showed a biphasic decline with an initial phase lasting for the first 120 min followed by a second slower phase. The half-life of the β-phase was 187 min. N-Desmethyl-clobazam was detected 5 min after administration of clobazam reaching peak concentrations at 60 min. The half-life of the metabolite was 225 min.

In conclusion the specificity and sensitivity of this procedure appear to be satisfactory for pharmacokinetics studies with clobazam. Findings in this laboratory indicate that the method can be extended to other animal species and to various organs after tissue homogenization.

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