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Note

Thin-layer chromatographic method for determination of diazepam and its major metabolite, N-desmethyldiazepam, in human serum

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Several methods have been reported for the determination of diazepam and its major metabolite, N-desmethyldiazepam, including gas chromatography with flame ionization[1,2] or electron-capture detection[3,4], high-performance liquid chromatography (HPLC)[5] and thin-layer chromatography (TLC)[6]. In this laboratory a gas chromatographic method in which diazepam and its metabolite are extracted and hydrolysed to their corresponding benzophenones has been used[2]. However, this method was found to be too time-consuming for use in a bioavailability study, and, since, we had much experience of quantitative *in situ* densitometry, we decided to develop such a procedure for diazepam and its metabolite.

MATERIALS AND METHODS

Reagents

The following reagents were used: diazepam and N-desmethyldiazepam (each from Hoffmann-La Roche, Basle, Switzerland); carbamazepine (Ciba-Geigy, Johannesburg, South Africa); and 0.1 *M* phosphate buffer of pH 8. All solvents and chemicals were of reagent grade.

Apparatus

A Perkin-Elmer MPF 3 spectrofluorimeter equipped with a xenon lamp and a scanning attachment for TLC was used. Chromatograms were irradiated with a universal UV-lamp, type TL-900/U (Camag, Muttenz, Switzerland). For TLC, we used silica gel 60 plates (10 × 20 cm) (Merck, Darmstadt, G.F.R.).

Standard solutions

A solution containing 5 ng/μl of both diazepam and N-desmethyldiazepam was prepared in methanol. As standard, we used a solution of carbamazepine (10 ng/5 μl) in methanol.

Extraction

To 1 ml of serum were added 1 ml of phosphate buffer (pH 8), 60 ng of carbamazepine (internal standard) and 5 ml of *n*-hexane containing 10% of isobutanol, the mixture was then shaken for 15 min on a mechanical shaker, and the organic phase was separated by centrifugation at 2000 *g*, and transferred to a 10-ml ampoule, where it was evaporated to dryness under a stream of air at 50°.

Spotting

The residue was dissolved in 60 μl of chloroform, and 10 μl of this solution were spotted on the TLC plate in two portions (5 μl each) using a disposable glass capillary; the liquid was allowed to run on to the plate by gravity and the natural capillary action of the plate.

Separation of the compounds was effected by ascending chromatography, in an unsaturated TLC chamber, the solvent [ethyl acetate–benzene–ammonia (35:15:0.15)] being allowed to migrate to a height of 6 cm. After drying, the plates were exposed for 30 min to hydrogen chloride gas generated in a chromatographic tank by pipetting 2 ml of concentrated hydrochloric acid into 10 ml of concentrated sulphuric acid in a beaker in the tank. Immediately after exposure to the gas, the plates were exposed for 45 min to radiation at 254 nm. This procedure converted the diazepam, the metabolite and the internal standard into fluorescent compounds, and the fluorescence was enhanced by dipping the plates in a 10% (w/v) solution of paraffin wax in light petroleum[7].

The intensity of fluorescence of the different compounds was measured quantitatively, using the following operating conditions: excitation wavelength, 360 nm; excitation slit-width, 10 nm; emission wavelength, 460 nm; scanning speed, high (4 cm/min); chart speed, low (2.4 cm/min). The emission slit-width, amplifier sensitivity, zero suppression and sample adjustment were so set as to obtain approximately 80% of full-scale deflection on the recorder when the most intense spot on the plate was being scanned.

RESULTS AND DISCUSSION

Quantitation of diazepam and N-desmethyldiazepam

Diazepam in concentrations ranging from 10 to 500 ng/ml in drug-free human serum was extracted after the addition of 60 ng of carbamazepine as internal standard as described above. Peak-height ratios of diazepam to internal standard were calculated, and, from the results, the best straight-line fit was obtained by linear regression analysis. Fig. 1 shows a typical scan, and Fig. 2 shows the standard graph for diazepam (ranging from 50 to 500 ng/ml).

For each series of determinations, a two-point calibration was used, consisting of duplicate extractions at two concentrations (100 and 300 ng/ml) of diazepam. The peak-height ratios were determined, a straight line was drawn through the points, and the equation thus derived was used to determine diazepam in sample sera. Determination of N-desmethyldiazepam was achieved by the same procedure, using the peak-height ratios of N-desmethyldiazepam to internal standard; Fig. 3 shows the standard graph for the metabolite (50 to 300 ng/ml).

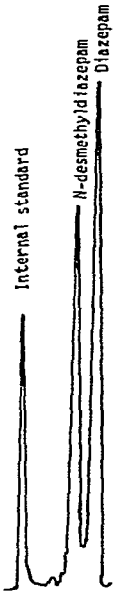

Fig. 1. Typical scan for 200 ng/ml each of diazepam and its metabolite.

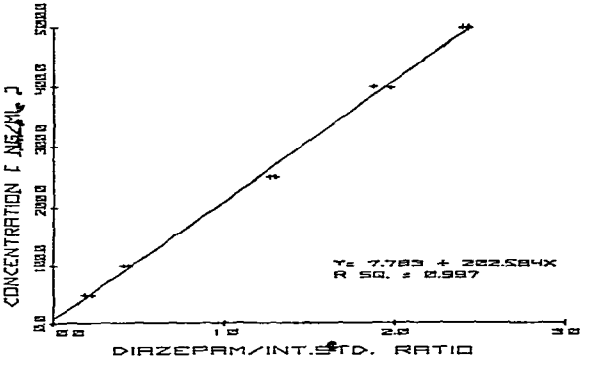

Fig. 2. Standard graph for diazepam.

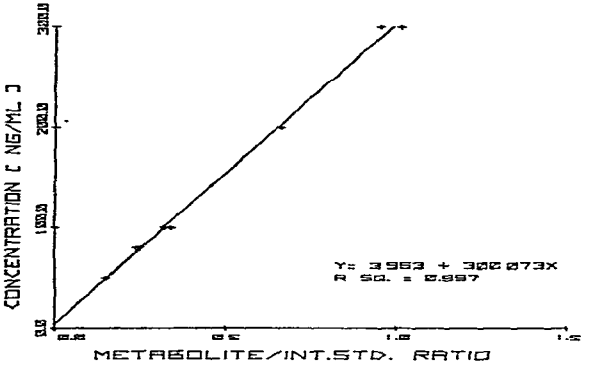

Fig. 3. tandard graph for N-desmethyldiazepamS.

TABLE I

RECOVERY OF DIAZEPAM AND N-DESMETHYLDIAZEPAM FROM SERUM

Amount added (ng/ml)		*Amount recovered (ng/ml)*		*Mean ± s.d. (ng/ml)*	
Diazepam	*N-Desmethyl-diazepam*	*Diazepam*	*N-Desmethyl-diazepam*	*Diazepam*	*N-Desmethyl-diazepam*
80	80	85.9	91.0		
		83.0	83.8		
		83.4	78.5		
		76.2	84.8	82.7 ± 3.4	82.5 ± 5.8
		86.0	76.5		
		84.0	87.2		
		80.7	75.5		
150	150	152.9	161.1		
		166.6	161.1		
		156.4	151.5		
		164.6	136.7	157.2 ± 6.9	151.6 ± 8.3
		160.8	149.8		
		149.7	151.5		
		149.7	149.8		
200	200	205.7	214.0		
		212.1	209.7		
		200.4	198.1		
		211.1	202.7	205.4 ± 8.2	200.7 ± 9.0
		215.3	193.5		
		191.5	198.7		
		201.9	188.1		

Recovery of diazepam and N-desmethyldiazepam

The recovery of diazepam and its metabolite was determined by preparing a calibration graph and analyzing "blind" serum samples that had been spiked with the two substances at levels from 10 to 500 ng/ml; the results are shown in Table I.

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