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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BENZODIAZEPINES IN HUMAN PLASMA

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SUMMARY

A simple and sensitive method for determination of benzodiazepines in plasma has been developed using high-performance liquid chromatography in a reverse-phase mode. The method is illustrated by application to plasma samples containing diazepam and N-desmethyldiazepam at concentrations which would be encountered during therapy, with limits of detection of 10 ng/ml and 2 ng/ml for diazepam and N-desmethyldiazepam, respectively.

INTRODUCTION

Diazepam, 1-methyl-1,3-dihydro-5-phenyl-7-chloro-2H-1,4-benzodiazepin-2-one, is a member of the benzodiazepine group of drugs widely used for the symptomatic relief of tension and anxiety states in man[1]. The metabolism of diazepam in humans is well documented[2–7]. N-Demethylation and hydroxylation represent major pathways of biotransformation, N-desmethyldiazepam being the main circulating metabolite.

Many papers have been published on the measurement of diazepam and its metabolites in biological fluids, gas chromatography (GC) being used extensively (see reviews by Hailey[8] and by Clifford and Franklin Smyth[9]). The GC methods require somewhat lengthy clean-up procedures and, in some cases, derivatisation or acid hydrolysis to the more volatile benzophenones.

High-performance liquid chromatography (HPLC) for the measurement of benzodiazepines and their metabolites involves relatively simple extraction, no derivatisation and UV detection to give high sensitivity, long-term stability and linearity over wide concentration ranges, and thus is useful for the measurement of concentrations of these drugs in therapeutic as well as toxic ranges.

The separation of diazepam and its metabolites by HPLC was reported by Scott and Bommer[10] using adsorption chromatography for the measurement of diazepam and metabolites at the microgram level in dog urine, and by Bugge[11] for the purpose of whole blood analysis in forensic toxicology, again using adsorption chromatography. More recently Uihlein and Hadju[12] have exemplified the use of

HPLC for the analysis of fosazepam and Harzer and Barchet[13] have used HPLC as a confirmatory technique to GC for the forensic determination of a series of benzodiazepines and their hydrolysis products in whole blood and urine.

This paper demonstrates the use of HPLC in a reverse-phase mode to separate diazepam from its major metabolite N-desmethyldiazepam in human plasma samples to obtain pharmacokinetic data. An internal standardisation technique was employed using a structurally related benzodiazepine (prazepam) as the internal standard.

EXPERIMENTAL

Materials

Reagents were of analytical grade, and inorganic reagents were prepared in freshly glass-distilled water. Diethyl ether was redistilled before use. Borate buffer (1 *M*) containing potassium chloride (1 *M*) was adjusted to pH 9.0 with sodium carbonate solution (1 *M*). Standard solutions of diazepam, N-desmethyldiazepam were prepared in methanol at concentrations of 1 μg/ml and 10 μg/ml, and prazepam (the internal standard, 1-cyclopropylmethyl-1,3-dihydro-5-phenyl-7-chloro-2H-1,4-benzodiazepin-2-one) at 10 μg/ml. Prazepam was kindly provided by Mr. J. C. Deavin of William R. Warner & Co. (Eastleigh, Great Britain).

Extraction

Plasma samples (2 ml) were spiked with internal standard (400 ng), mixed with borate buffer (2 ml) and adjusted to pH 9.0 if necessary with sodium hydroxide (4 *M*). This mixture was extracted by shaking it with diethyl ether (10 ml) for 10 min. After centrifugation, the ether phase was transferred to a 10-ml pointed tube and evaporated to dryness under a stream of nitrogen at 35°. The residue was washed to the bottom of the tube with a small volume of diethyl ether, which was evaporated to dryness. The residue was redissolved in methanol (25 μl) and a portion (10–20 μl) was injected into the chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M6000A pump (Waters Assoc., Cheshire, Great Britain) fitted to a fixed-wavelength (254 nm) UV monitor operated at a maximum sensitivity of 0.02 a.u.f.s. Injection was made by syringe via a U6K universal injector (Waters Assoc.). The column was 30 cm × 4 mm I.D., prepacked with μBondapak C18 (Waters Assoc.). Chromatography was performed in reverse-phase mode using a solvent system of methanol–water (65:35, v/v) at a flow-rate of 2 ml/min. Under these conditions, diazepam, N-desmethyldiazepam and the internal standard, prazepam were eluted with retention times of 6.0, 5.0 and 10.5 min, respectively (Fig. 1).

Collection of samples

Blood samples were withdrawn from five male volunteer human subjects who were participating in pharmacokinetic studies of diazepam. The subjects remained under medical supervision during the period of the study and were in good health. Each subject received a dose of 5 mg diazepam at 24-h intervals during 10 consecutive days. Blood samples were withdrawn into heparinized tubes before dosing com-

menced, and at 12 h after each daily dose. Further samples were withdrawn during 15 days after the last dose. Blood cells were removed by centrifugation and the separated plasma was stored at −20°.

RESULTS AND DISCUSSION

Concentrations of diazepam and N-desmethyldiazepam were determined from calibration curves constructed by plotting the ratio of peak height measurements of drug and metabolite to the internal standard (200 ng/ml) over the concentration range 10–400 ng/ml. The recovery of internal standard from plasma was 96 ± 3% [standard deviation (S.D.); $n = 5$]. The overall recovery of diazepam (96 ± 3%) and N-desmethyldiazepam (94 ± 4%) over the concentration range 10–400 ng/ml was calculated by comparing peak height ratio measurements of standards to those of standards extracted from plasma and corrected for losses of internal standard. The calibration curves (Fig. 2) were constructed from five replicate measurements of six concentrations over the range and plots of peak height ratios against concentration were linear: $y = a + bx$, where a = 0.0095 ± 0.0087 (S.D.), b = 0.0089 ± 0.00005 (S.D.) for diazepam, and a = 0.0011 ± 0.0074 (S.D.), b = 0.0101 ± 0.00004 (S.D.) for N-desmethyldiazepam; the value of the intercept was not significantly different from zero. The 95% confidence limits of the least squares regression line forced

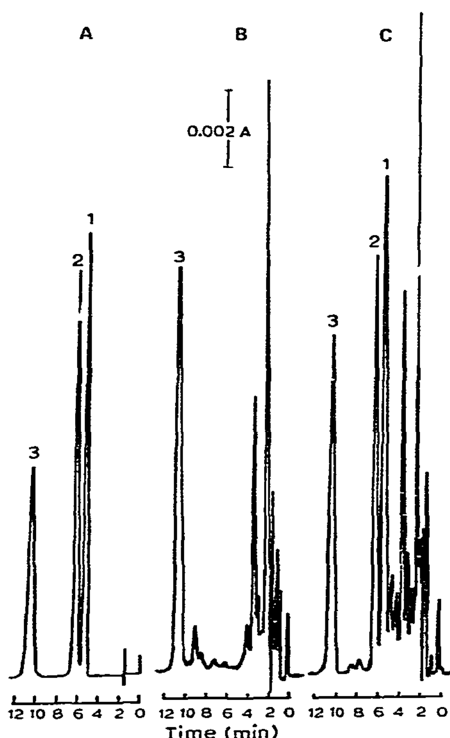
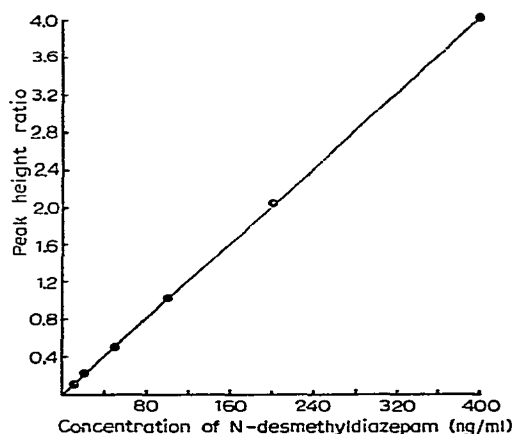


Fig. 1. (A) Chromatogram of benzodiazepine standards: 1 = N-desmethyldiazepam, 2 = diazepam, 3 = prazepam (internal standard). (B) Blank control plasma extract containing internal standard only. (C) Human plasma extract containing N-desmethyldiazepam (137 ng/ml) and diazepam (126 ng/ml).



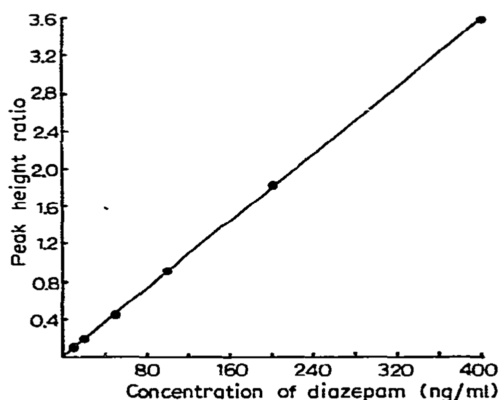


Fig. 2. Calibration curves of N-desmethyldiazepam and diazepam.

TABLE I

MEAN PLASMA CONCENTRATIONS OF DRUG AND METABOLITE 12 h AFTER DOSING, DURING A PERIOD OF 10 CONSECUTIVE DAILY ORAL DOSES OF DIAZEPAM TO 5 HUMAN SUBJECTS

Concentrations are presented together with S.D. values for $n = 5$.

Time (days)	*Diazepam conc. (ng/ml)*	*N-Desmethyldiazepam conc. (ng/ml)*
1	58 ± 14	16 ± 5
2	93 ± 26	47 ± 11
3	98 ± 36	70 ± 12
4	103 ± 22	92 ± 15
5	105 ± 34	110 ± 25
6	108 ± 30	122 ± 28
7	101 ± 20	130 ± 28
8	103 ± 35	138 ± 35
9	116 ± 35	143 ± 33
10	104 ± 39	131 ± 44

through the origin were ± 80% at 10 ng/ml, ± 4% at 200 ng/ml and ± 2% at 400 ng/ml for diazepam, and ± 59%, ± 3% and ± 2% at concentrations of 10, 200 and 400 ng/ml, respectively, for N-desmethyldiazepam.

No interfering peak with the retention time of N-desmethyldiazepam was present in extracts of "blank" control plasma. The limit of detection of N-desmethyldiazepam was therefore approximately 2 ng/ml, allowing a signal-to-noise ratio of 2:1. An interfering peak with the same retention time of diazepam was present in extracts of blank control plasma, and which was equivalent to drug concentrations of 4 ± 2.5 (S.D.) ng/ml. The upper 95% confidence limit of the mean diazepam blank (10 ng/ml) was considered to be the limit of detection of concentrations of drug in plasma.

When applied to the collected plasma samples, the method showed that plasma concentrations of diazepam and N-desmethyldiazepam increased during the period of dosing to reach plateau levels of 100–110 ng/ml diazepam and 120–140 ng/ml N-desmethyldiazepam after 6 days (Table I). After the last dose, plasma concentrations of both drug and metabolite decreased apparently logarithmically (Fig. 3) with half-lives of 21 h and 53 h for diazepam and N-desmethyldiazepam, respectively, which are somewhat shorter than those reported elsewhere[14,15].

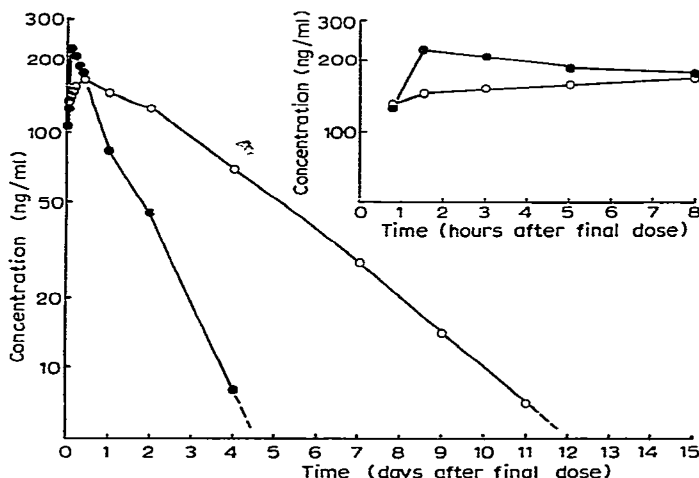


Fig. 3. Mean plasma concentrations of (○) N-desmethyldiazepam and (●) diazepam after the final dose (semi-logarithmic scale).

This method, with minor modifications, has also been used successfully to measure concentrations of benzodiazepines in other biological fluids, such as whole blood and milk. For the analysis of complex biological extracts, the use of HPLC in a reverse-phase mode of operation ensures a longer column life since polar materials are not retained on the column, as they are by adsorption chromatography. No appreciable loss in resolution was observed during the chromatography of more than 500 sample extracts and peak height ratio measurements of standards routinely measured had coefficients of variations of less than ±1%.

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