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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LORAZEPAM IN MONKEY PLASMA

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SUMMARY

A simple, sensitive and selective method for the determination of lorazepam in monkey plasma has been developed using high-performance liquid chromatography in a reversed-phase mode. The limit of detection for lorazepam in plasma is about 2 ng/ml. The method has been applied to plasma samples obtained from cynomolgus monkeys after oral doses of 0.15 mg/kg and intravenous doses of 0.05 mg/kg of lorazepam. In this species, mean peak plasma concentrations of 12 ng/ml occurred at 2 h after oral dosing and declined with a half-life of 2.5 h; the mean terminal half-life after intravenous dosing was 1.4 h.

INTRODUCTION

Lorazepam[7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one] (Fig. 1) is a member of the benzodiazepine group of anxiolytic drugs. It is currently used as a premedicant in anaesthesia [1, 2] and for the relief of anxiety states in man [3, 4]. Lorazepam is extensively metabolised in the rat, but in man and other investigated species there is only one major metabolite, lorazepam glucuronide [5, 6], which has no psychopharmacological activity [7].

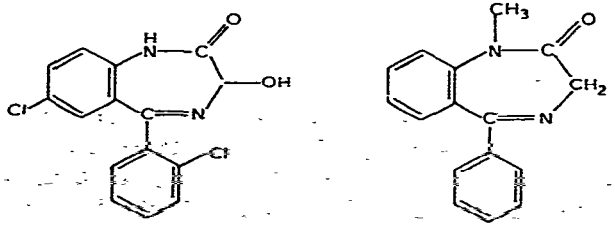

Fig. 1. Structures of lorazepam (left) and internal standard (diazepam) (right).

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For accurate pharmacokinetic studies of drugs in biological fluid, the analytical techniques employed should discriminate between the parent drug and its metabolites and should quantify them at levels normally encountered during drug therapy. Early gas chromatographic (GC) methods employed for the analysis of lorazepam either lacked specificity as a result of utilising acid hydrolysis to the benzophenone, or sensitivity due to on-column adsorption phenomena [8]. More recent GC methods require minimal clean-up procedures, require no derivatisation and are specific for lorazepam [9, 10].

This paper describes a rapid, sensitive and selective assay for lorazepam in monkey plasma using high-performance liquid chromatography (HPLC) in a reversed-phase mode. The system is non-destructive and uses an internal standardisation technique employing the benzodiazepine, diazepam, as the internal standard. In contrast to a recent review [11] which considered HPLC to be insufficiently sensitive as a technique for the quantitation of therapeutic concentrations of lorazepam, this paper demonstrates that HPLC can be applied to pharmacokinetic studies in monkeys at dose levels which are within the human therapeutic range.

EXPERIMENTAL

Materials

Acetonitrile was HPLC (far UV) grade. All other reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Diethyl ether was redistilled prior to use. Borate buffer (1 *M*) containing potassium chloride (1 *M*) was adjusted to pH 9.0 using sodium carbonate solution (1 *M*). Standard solutions of lorazepam were prepared in methanol at concentrations of 1 μg/ml and 10 μg/ml; a stock solution of the internal standard, diazepam, was prepared at a concentration of 10 μg/ml.

Extraction

Plasma samples (1 ml) were spiked with internal standard (100 ng) and mixed with borate buffer (1 ml) to adjust the pH to 9.0. The samples were extracted by vortex mixing with diethyl ether (5 ml) for 30 sec. After centrifugation, the ether phase was transferred to a 10-ml pointed centrifuge tube and evaporated to dryness under a stream of dry nitrogen at 37°C. The residue was washed to the bottom of the tube with a small volume of diethyl ether, which was again evaporated to dryness. The residue was redissolved in 100 μl of acetonitrile—water (50:50, v/v) and half of the sample (50 μl) was injected into the liquid chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M6000A pump (Waters Assoc., Cheshire, Great Britain) fitted to a Pye LC3 variable-wavelength UV absorption detector (Pye Unicam, Cambridge, Great Britain) operated at 230 nm (a λ_{max} for lorazepam in methanol). Injection was via an automatic sampler WISP™ 710A (Waters Assoc.).

The column was constructed of stainless steel (30 cm × 0.4 cm I.D.) prepacked with μBondapak C_{18} (mean particle diameter 10 μm) (Waters Assoc.). A

pre-column (7 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 27—37 μm, Whatman, Kent, Great Britain) was installed in front of the main analytical column.

Chromatography was performed in a reversed-phase mode using a solvent system of 40% (v/v) acetonitrile in aqueous 0.1% (w/v) sodium dihydrogen orthophosphate, the final pH of the mobile phase was adjusted to 3.0 with phosphoric acid. The mobile phase flow-rate was 2 ml/min.

Chromatograms were recorded using either a Trilab computing integrator (Trivector Systems, Sandy, Great Britain) or a 3380A computing integrator (Hewlett-Packard, High Wycombe, Great Britain). Peak height measurements were used in preference to peak area measurements since these gave greater precision of measurement.

Under the conditions described, lorazepam and the internal standard (diazepam) eluted from the column with retention times of 4.3 and 8.8 min, respectively (Fig. 2).

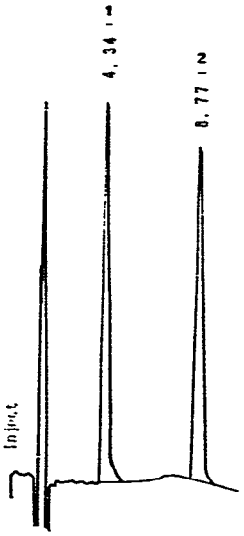

Fig. 2. Chromatogram of a standard mixture containing lorazepam (1) and internal standard, diazepam (2). Column: 30 × 0.4 cm I.D. prepacked with μBondapak C_{18}; flow-rate, 2 ml/min; solvent system, 40% (v/v) acetonitrile—aqueous 0.1% (w/v) sodium dihydrogen orthophosphate; detector, UV, at 230 nm; integrator attenuation, 16.

Calibration procedure

Calibration lines of peak height ratio measurements of lorazepam to internal standard against concentrations of lorazepam (ng/ml) were constructed over the concentration range 0—100 ng/ml. Samples of blank (drug-free) plasma (1 ml), and plasma spiked with lorazepam at concentrations of 5, 10, 30, 50, 70 and 100 ng/ml and with internal standard at a fixed concentration of 100 ng/ml, were taken through the extraction procedure described previously. Five replicate extractions were made at each concentration over the calibration range.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out using a Pye 104 gas chromatograph (Pye-Unicam) linked via a single-stage, glass jet separator to a Micromass 16F mass spectrometer (V.G. Analytical, Cheshire, Great Britain). The mass spectrometer was operated in the electron impact mode of ionisation with an electron energy of 70 eV, a trap current of 100 μA and a source temperature of 200°C. Mass spectra were obtained at 10-sec intervals and the data stored using a Display Digispec data system (V.G. Analytical) on floppy diskettes.

The gas chromatograph oven was fitted with a glass column (15 m × 0.2 cm I.D.) packed with 3% OV-17 on Diatomite CC1 (100—120 mesh) and was operated at 260°C. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The temperature of the GC—MS interface was 250°C.

Lorazepam samples were derivatised with N,O-bis(trimethylsilyl)acetamide in pyridine [Trisil/BSA Formula 'P'; Pierce and Warriner (U.K.), Cheshire, Great Britain]. Sample residues (ca. 1—10 μg) were heated with the derivatising agent (10—20 μl) for 15 min at 40°C; aliquots containing 0.5—1 μg of lorazepam were injected for GC—MS. Under the above conditions the bis(trimethylsilyl) derivative of lorazepam was formed and gave a retention time of 5 min.

Studies in the cynomolgus monkey (Macaca fascicularis)

Five adult male cynomolgus monkeys were each given single oral doses of 0.15 mg lorazepam per kg bodyweight and 0.05 mg/kg intravenous doses. The oral doses were administered in 10 ml water via a stomach tube and were washed in with an equal volume of water. The animals were fasted for 12 h preceding drug administration and for 6 h following drug administration.

Blood samples (3—5 ml) were withdrawn from the femoral veins of the animals into heparinised tubes, at 0 h (predose) and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 7 and 24 h after dosing; an additional sample was taken at 2 min after intravenous dosing. Blood cells were removed by centrifugation and the separated plasma was analysed immediately.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration were repeated on five occasions. The precision of the method for the measurement of lorazepam in plasma as indicated by the coefficient of variation of peak height ratio measurements was ± 8% at 5 ng/ml, ± 2% at 30 ng/ml and ± 2% at 100 ng/ml (Table I).

Accuracy

A calibration line for the measurement of lorazepam in plasma, constructed over the concentration range 0—100 ng/ml was linear ($y = -0.014 + 0.0126x$, correlation coefficient 0.9958) where y is the peak height ratio and x the concentration of lorazepam in plasma (ng/ml). However, two overlapping calibration lines were constructed for the ranges 0—50 ng/ml ($y = -0.004 + 0.0118x$, correlation coefficient 0.9943) and 30—100 ng/ml ($y = -0.048 + 0.0131x$, correlation coefficient 0.9891). The use of two calibration lines improved the

TABLE I

PRECISION OF THE METHOD AND RECOVERIES OF LORAZEPAM FROM PLASMA

Values corrected for 100% recovery of internal standard.

Concentration added to plasma (ng/ml)	Coefficient of variation (%)	Recovery (%)
5	8	66
10	9	71
30	2	69
50	6	71
70	2	75
100	2	78

accuracy of measurement in the lower regions of the calibration range; clinical doses of lorazepam produce plasma concentrations of the drug of this order of magnitude. The standard error of the calibration line as a measure of the concentration of lorazepam in plasma was 2.25 ng/ml.

Recovery

The recovery of internal standard from plasma (100 ng/ml) was 77 ± 6% S.D. (n = 6). The mean recovery of lorazepam over the concentration range 5—100 ng/ml was determined by comparison of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was 72 ± 4% S.D. (Table I).

Limit of detection

No interfering peaks with the same retention time as lorazepam were present in the predose (blank) plasma samples taken from the cynomolgus monkeys. The limit of accurate measurement of the method based on a signal-to-noise ratio of 2:1 was set at 5 ng/ml (Fig. 3). Since, however, the extraction method described could also be used for the extraction of 2 ml of plasma without deviation in peak height ratios from the calibration line based on 1 ml of plasma, it was also possible to measure 5 ng of lorazepam in 2 ml of plasma giving a limit of detection of the order of 2—3 ng/ml. This limit of detection is of the same order of magnitude as that of the reported GC methods [9, 10].

Selectivity of the analytical method

The selectivity of the analytical method was determined by GC—MS. The mass spectra for the bis(trimethylsilyl) derivatives of the authentic drug, and lorazepam separated by HPLC from the plasma of treated cynomolgus monkeys, were virtually identical (Fig. 4). The spectrum of the derivatised compound had a molecular ion at m/e 464 and characteristic fragments at m/e 449 (M — CH_3) and m/e 429 (M — Cl) all showing the expected isotope pattern of Cl-containing ions.

Concentrations of lorazepam in monkey plasma

After single oral doses of lorazepam to cynomolgus monkeys, a peak of mean concentrations of 12 ng/ml was reached at 2 h after dosing (Table IIa).

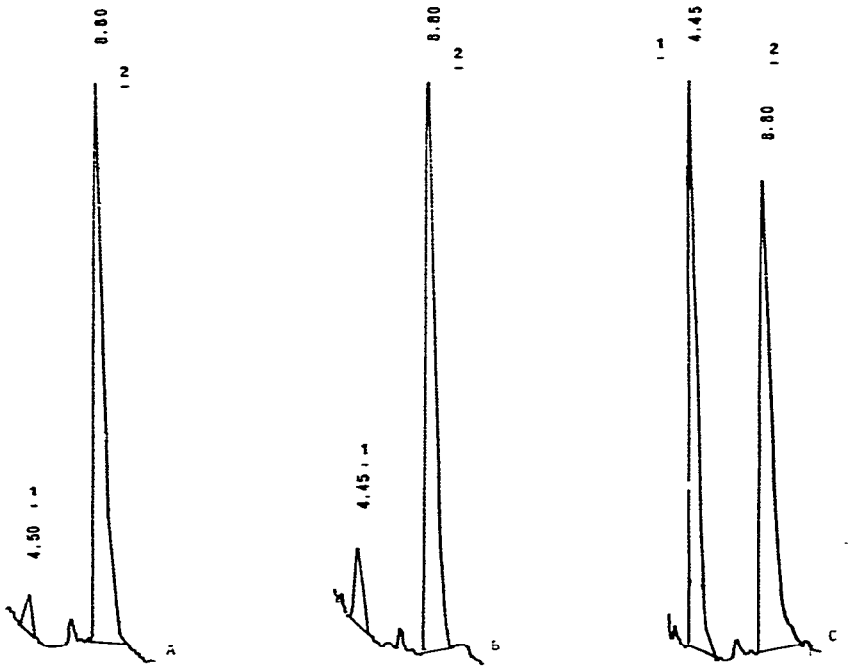

Fig. 3. Chromatograms of plasma standards containing (A) 5 ng/ml, (B) 10 ng/ml and (C) 100 ng/ml lorazepam. Conditions as for Fig. 2. Peaks: 1 = lorazepam, 2 = internal standard.

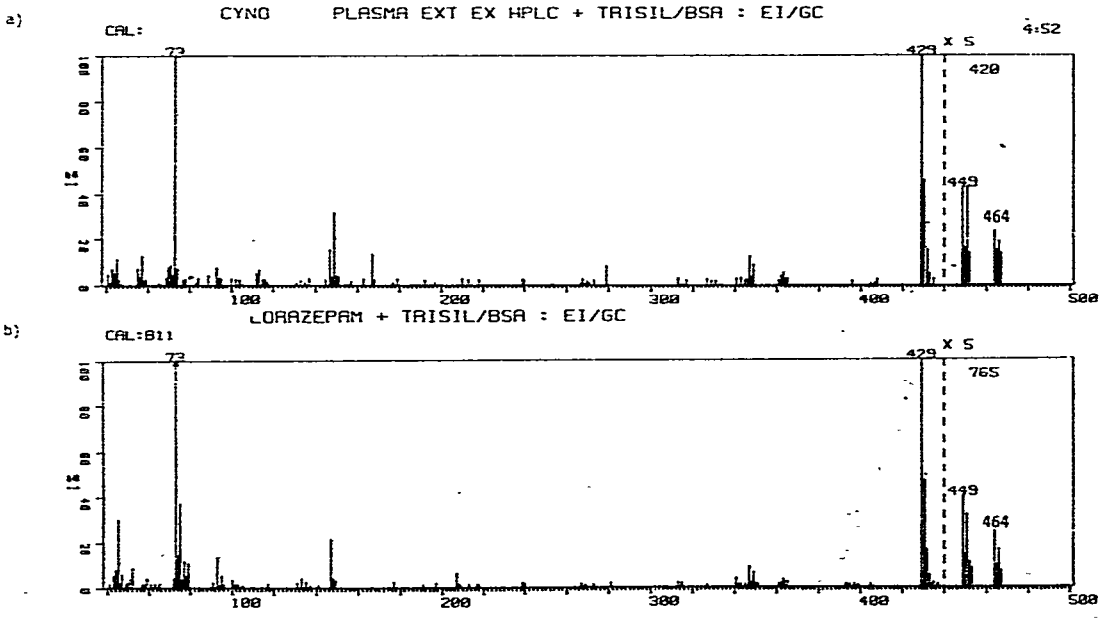

Fig. 4. Mass spectra of the trimethylsilyl derivatives of (a) lorazepam separated by HPLC from the plasma of a treated cynomolgus monkey and (b) authentic drug.

TABLE II

PLASMA CONCENTRATIONS OF LORAZEPAM (ng/ml) IN CYNOMOLGUS MONKEYS

Time (h)	Monkey number					Mean ± S.D.
	1	2	3	4	5	
(a) After single oral doses of 0.15 mg/kg						
0.25	4	6	6	4	6	5 ± 1
0.50	8	8	7	10	11	9 ± 2
0.75	8	7	10	8	16	10 ± 4
1.00	11	11	9	9	15	11 ± 2
1.50	8	7	9	15	17	11 ± 4
2.00	7	15	8	13	17	12 ± 4
3.00	4	12	8	9	11	9 ± 3
4.00	3	8	7	8	8	7 ± 2
5.00	<2	5	5	7	7	5 ± 3
7.00	2	5	<2	5	3	3 ± 2
(b) After single intravenous doses of 0.05 mg/kg						
0.03	46	84	24	78	74	61 ± 25
0.25	34	34	27	73	64	46 ± 21
0.50	22	28	23	56	48	35 ± 16
0.75	18	20	17	42	39	27 ± 12
1.00	12	16	14	37	31	22 ± 11
1.50	9	13	10	22	25	16 ± 7
2.00	8	10	9	20	18	13 ± 6
3.00	4	7	6	13	11	8 ± 4
4.00	<2	6	4	5	8	5 ± 3
5.00	<2	8	3	4	4	4 ± 3
7.00	<2	2	<2	<2	<2	<2

Thereafter mean concentrations declined to 3 ng/ml at 7 h after dosing. The mean half-life of lorazepam in the plasma of cynomolgus monkeys after 0.15 mg/kg oral doses was 2.5 h. This is markedly shorter than the half-life of the drug in human plasma (12—14 h) [12, 13] after single oral doses. The dose applied to the cynomolgus monkeys was three times the human clinical dose but rapid elimination of lorazepam by the cynmolgus monkey precluded accurate assessment of pharmacokinetic parameters at lower dose levels by this route of administration.

Plasma concentrations of lorazepam in the same monkeys after 0.05 mg/kg intravenous doses of the drug are shown in Table IIb. Mean plasma concentrations of lorazepam declined with a terminal half-life of 1.4 h. Representative plots of lorazepam concentrations in the plasmas of three cynomolgus monkeys after oral and intravenous doses are shown in Fig. 5. Plasma concentrations during the terminal β-phase after intravenous doses of lorazepam to monkeys are of the same order of magnitude as would be encountered after single therapeutic doses of lorazepam to humans.

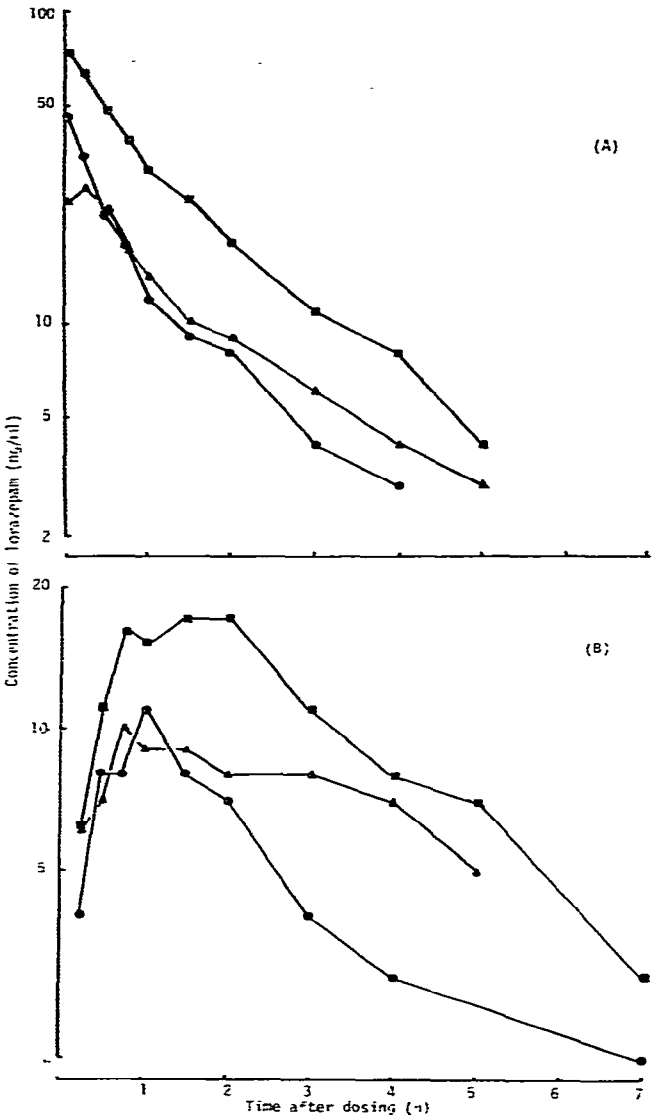

Fig. 5. Representative plots of concentrations of lorazepam in the plasma of cynomolgus monkeys after (A) an intravenous dose of 0.05 mg/kg and (B) an oral dose of 0.15 mg/kg. Semi-logarithmic scale. Symbols: ● = animal No. 1; ▲ = animal No. 3; ■ = animal No. 5.

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