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Note

High-performance liquid chromatographic determination of bromazepam in human plasma

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Since the early 1960s, benzodiazepines have been widely used as minor tranquillizers, sleep inducers and muscle relaxants. Bromazepam is a member of the 1,4-benzodiazepine class of compounds and was synthesized by Fryer et al. [1]. A usual daily therapeutic dose of bromazepam is 3—8 mg. Such a low therapeutic dose is associated with the strong action of this drug and results in low concentrations in plasma.

Analytical methods such as gas—liquid chromatography (GLC) [2—4] and thin-layer chromatography (TLC) [5] have been reported for determining bromazepam and its metabolites in body fluids. There has been no literature available concerning the determination of bromazepam in the plasma or serum by high-performance liquid chromatography (HPLC). It was the aim of this work to establish the assay method for the quantitation of bromazepam in human plasma by HPLC. The method will be applied for pharmcokinetic and bioavailability study of the drug.

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EXPERIMENTAL

Reagents

Reagents used were of analytical grade. Bromazepam [7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one] was obtained from Kodama (Tokyo, Japan). Tetra-*n*-butylammonium hydroxide (TBAH) 10% in water was obtained from Tokyo Kasei Industries (Tokyo, Japan).

Borate buffer (1 *M*) was adjusted to pH 8.0 with sodium hydroxide (0.1 *M*). Standard solutions of bromazepam were prepared in methanol at concentrations of 10.3 and 103.0 ng/ml. A methanol solution of carbamazepine (5H-dibenzo[*b*,*f*]azepine-5-carboxamide) was prepared at a concentration of 51.0 ng/ml and used as the internal standard.

Mobile phase

Twenty milliliters of 10% TBAH in water were added to 1 l of water and the pH was adjusted to 7.5 with phosphoric acid. The mobile phase was prepared by adding 700 ml of the above TBAH solution and 20 ml of methanol to 300 ml of acetonitrile.

High-performance liquid chromatography

The chromatograph consisted of a Hitachi 638-30 fitted to a variable-wavelength UV monitor, Hitachi 635-0900, operated at a maximum sensitivity of 0.005 a.u.f.s. (230 nm). Injection was made by a 50-μl micro-syringe via a Hitachi 638-0801 injector. The column was 30 cm × 4 mm I.D., prepacked with μBondapak C_{18}, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.). Chromatography was performed in reversed-phase mode at a flow-rate of 1 ml/min at room temperature. The chart speed was 5 mm/min.

Extraction procedure

One milliliter of plasma, 1 ml of internal standard solution, 2 ml of borate buffer (pH 8.0), and 20 ml of toluene were combined in a glass-stoppered extraction tube. The tube was then shaken for 10 min. After centrifugation, the toluene phase was transferred to a flask and evaporated to dryness under reduced pressure.

The residue was allowed to stand at −20° C for 2 h and redissolved with 3 ml of ethanol—water (90:10, v/v). The solution was transferred to a 10-ml tube. The tube was again allowed to stand at −20° C for 2 h. The ethanol solution was then centrifuged at 1500 *g* for 10 min and decanted into a 10-ml flask and evaporated to dryness under reduced pressure. The residue was resuspended in 100 μl of acetonitrile—TBAH (60:40, v/v), pH 7.5, and a 40-μl portion was injected into the chromatograph.

RESULTS AND DISCUSSION

Direct GLC measurements of underivatized bromazepam [2, 3] or the TLC method [5] do not provide accurate and sensitive analysis of this compound in biological fluids. The methylation of bromazepam to its N^1-methyl derivative, however, results in a significant improvement of the GLC measurements

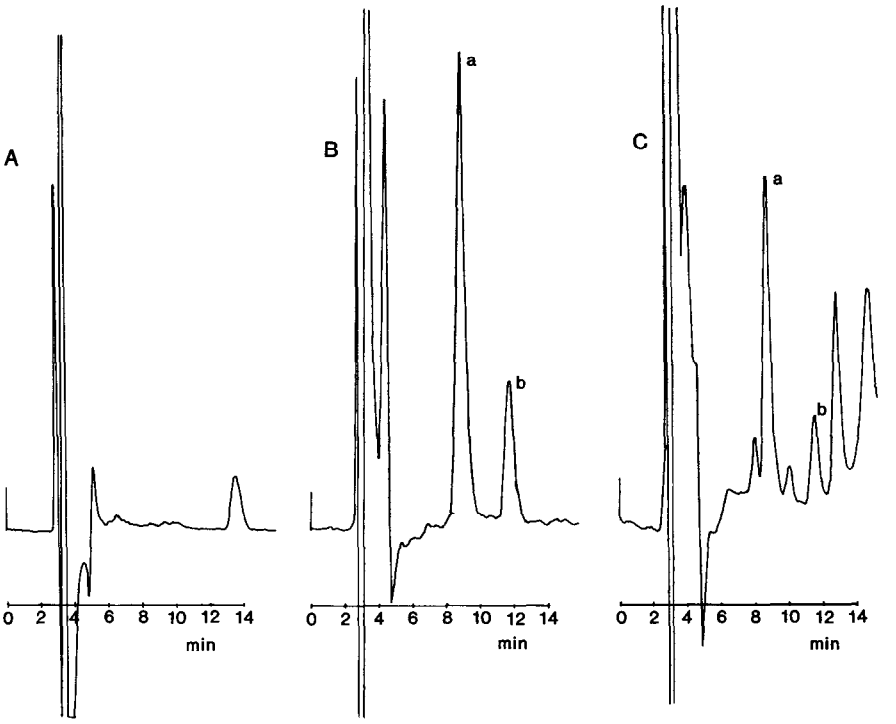

Fig. 1. (A) Chromatogram of an extracted blank plasma. (B) Chromatogram of an extracted standard plasma (1.0 ml) spiked with 103 ng of bromazepam (peak a) and 51 ng of internal standard (peak b). (C) Chromatogram of an extracted plasma (1.0 ml) from a patient dosed with a 5-mg bromazepam tablet.

and the method was successfully applied for pharmacokinetic studies in man [4]. The present HPLC method does not require a derivatization procedure and the sample can be directly injected onto the HPLC column after the initial extraction procedure.

A typical chromatogram of bromazepam, obtained after processing 1 ml of plasma spiked with 103.0 ng of the drug is shown in Fig. 1 together with chromatograms of an extracted blank plasma and a patient's sample. Peaks a and b correspond to bromazepam and the internal standard, respectively. The respective retention times are 8.5 and 11.5 min. There were no interfering peaks originating from endogenous compounds in plasma. Other drugs, such as neostigmine methyl sulfate, pentazocine, atropine sulfate and thiopental sodium, commonly used concomitantly with bromazepam, were found not to interfere with the bromazepam analysis.

Calibration curve

Calibration curves of plots of peak height ratios against concentrations were linear ($Y = 0.135 + 0.0271X$; $r = 0.999$) over the concentration range 10—200 ng/ml.

Analytical recovery

Known amounts (20.6, 51.5 and 103.0 ng) of bromazepam and a constant amount (51.0 ng) of internal standard were added to 1-ml portions of pooled plasma and the samples were processed as described. The analytical recovery of bromazepam at these concentrations was between 93.7 and 108.7% (Table I).

TABLE I

RECOVERY OF BROMAZEPAM FROM PLASMA

Drug added to 1 ml plasma (ng)	No. of determinations	Drug recovered (ng)	Recovery (%)	Mean recovery (%)	C.V.* (%)
20.6	1	19.3	93.7		
	2	20.6	100.0	98.4	4.72
	3	19.5	94.7		
	4	21.7	105.3		
51.5	1	50.8	98.6		
	2	48.8	94.8	101.8	5.31
	3	56.0	108.7		
	4	54.1	105.0		
103.0	1	111.6	108.3		
	2	102.1	99.1	103.1	4.70
	3	100.3	97.4		
	4	110.6	107.4		

*C.V. = coefficient of variation.

Reproducibility

Day-to-day reproducibility of the method was evaluated by analyzing a pooled plasma containing bromazepam in a concentration of 51.5 ng/ml on five different days. A calibration curve was prepared on each assay day. Each calibration curve was constructed by determining two low concentrations (10.3 and 20.6 ng/ml) and two high concentrations (103.0 and 206.0 ng/ml). The mean value for the drug concentration determined was 50.2 ng/ml and the coefficient of variation was 5.5%.

Precision at low concentrations

Plasma samples with three different low concentrations of bromazepam, 5.2, 10.3, and 20.6 ng/ml, were analyzed in one assay run. The results are summarized in Table II. The mean peak height ratio and the coefficient of variation at a concentration of 5.2 ng/ml were 0.248 and 5.6%, respectively. A strict linearity was observed at these concentrations and the method provided satisfactory precision at concentrations as low as 5 ng/ml.

TABLE II

PRECISION OF ASSAY METHOD AT LOW CONCENTRATIONS

Drug added to 1 ml plasma (ng)	Peak height ratio						C.V.* (%)
	Individual values				Mean	S.D.	
5.2	0.250	0.262	0.225	0.254	0.248	0.0138	5.6
10.3	0.369	0.379	0.436	0.452	0.409	0.0356	8.7
20.6	0.658	0.692	0.663	0.722	0.684	0.0256	3.7

*C.V. = coefficient of variation.

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