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DETERMINATION OF BROMAZEPAM IN PLASMA AND OF ITS MAIN METABOLITES IN URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of bromazepam in plasma and of its main metabolites in urine is described. The unchanged drug is extracted from plasma with dichloromethane, using Extrelut 1 extraction tubes. The residue from this extract is subsequently analysed by reversed-phase high-performance liquid chromatography with ultraviolet detection (230 nm). The limit of detection is 6 ng/ml of plasma, using a 1-ml specimen. For the determination of the metabolites, the urine samples are incubated to effect enzymatic deconjugation and are then extracted with dichloromethane. Following two clean-up steps (back extractions), the final residue is analysed on the same reversed-phase system as the plasma samples. The limit of detection for the two metabolites is 200 ng/ml.

INTRODUCTION

Bromazepam (I) is a member of the 1,4-benzodiazepine class of compounds and is in clinical use as an antianxiety agent. After both oral and intravenous administration the compound is almost completely metabolized [1, 2]. The major urinary metabolites are the conjugates of 3-hydroxybromazepam (II) and of 2-(2-amino-5-bromo-3-hydroxybenzoyl)pyridine (III). The parent drug (I) and 2-(2-amino-5-bromobenzoyl)pyridine (ABBP) (IV) are excreted only in small amounts (Fig. 1).

Several methods have been described for the determination of the parent drug. Gas—liquid chromatographic (GLC) determinations include the direct measurement of the unchanged drug [3], hydrolysis followed by quantification as 2-(2-amino-5-bromobenzoyl)pyridine (ABBP) [4, 5], and determination as the N^1-methyl derivative [6]. With thin-layer chromatography (TLC),

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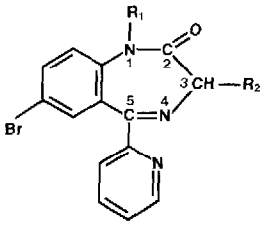
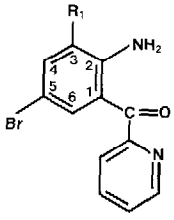
1,4-benzodiazepin-2-one



benzoyl-pyridine



Compound	R_1	R_2
Bromazepam [I]	H	H
3-Hydroxy-Bromazepam [II]	H	OH
N_1-Methyl-Bromazepam [V]	CH_3	H

Compound	R_1
ABBP [IV]	H
3-Hydroxy-ABBP [III]	OH

Fig. 1. Chemical structure of bromazepam, its metabolites, and the internal standards.

bromazepam can be determined either directly or after derivatization to an azo dye on the plate [7].

It was the aim of this work to develop a high-performance liquid chromatographic (HPLC) method for the determination of the unchanged drug in plasma and of the main metabolites in urine. Methylbromazepam (V) was used as internal standard.

EXPERIMENTAL

Reagents and materials

Dichloromethane, methanol, 3 *M* and 1 *M* sodium hydroxide and 3 *M* hydrochloric acid were all p.a. grade from E. Merck (F.R.G.). Phosphate buffers (Sörensen) were 0.067 *M* pH 7.5, 0.5 *M* pH 5, and 1 *M* pH 6; sodium acetate buffer was 0.2 *M* pH 5. Enzymes were Suc d'*Helix pomatia*, 100,000 units (Fishman) β-glucuronidase, 1,000,000 units (Roy) sulphatase (Pharmindustrie, France), and β-glucuronidase from bovine liver, 170,000 Fishman units/g (Serva, F.R.G.).

Solid extraction columns (glass) Extrelut 1 (Art. No. 15371, Merck) were used.

Chromatography

The following modular system was used: Waters M6000 HPLC pump (Waters Assoc., U.S.A.); Knauer Model 8700 UV—HPLC detector (Knauer, F.R.G.); automated sampling system ISS-100 (Perkin-Elmer, F.R.G.); Model 1100 W+W recorder (Kontron, Switzerland).

The column was a prepacked HPLC column Supelcosil LC 18, 5 μm, 15 cm × 4.6 mm (Supelco, Switzerland).

The mobile phase consisted of a mixture of methanol—0.067 *M* phosphate buffer pH 7.5 (47:53) at a flow-rate of 1 ml/min (room temperature, 70 bars). The monitoring wavelength was 230 nm, with a detector range setting of 0.04 a.u.f.s.

The following retention times (min) were obtained: 3-hydroxybromazepam

(II), 6.2; 3-hydroxy-ABBP (III), 9.0; bromazepam (I), 10.0; internal standard (V), 11.5; ABBP (IV), 19.5.

Solutions

Methanolic solutions (0.5 mg/ml) of compounds I, II, III and V (internal standard) were prepared. The bromazepam solution (I) was further diluted with water to a concentration of 0.05 mg/ml. From the solution of the internal standard (V), further dilutions in water were made to be used for internal standardization.

Plasma standards. A 200-μl volume of the aqueous solution of bromazepam (I) was added to drug-free plasma (50 ml). Starting from this stock plasma (200 ng/ml), the standards 100, 50, 25 and 12.5 ng/ml were prepared by stepwise dilutions with drug-free plasma.

Urine standards. A 200-μl volume of each of the methanolic solutions of II and III was added to drug-free urine (50 ml). Further dilutions from this stock urine (2000 ng/ml) with drug-free urine were made to obtain the concentrations 1500, 1000, 500 and 250 ng/ml.

Extraction of plasma samples

The extraction was carried out using solid extraction columns Extrelut 1. First, 1 *M* sodium hydroxide (100 μl), plasma (1 ml) and 100 μl of the aqueous solution of the internal standard, in that order, were applied to the column. Then the mixture was allowed to stand for 10 min and the drug was then extracted twice, each time with a 5-ml portion of dichloromethane. The extracts were combined in a conical glass tube and then evaporated to dryness under a gentle stream of nitrogen. The residue of the extract was redissolved in the eluent (200 μl) and injected for analysis (100 μl).

Extraction of urine samples

Urine (1 ml), buffer (1 ml), internal standard (100 μl of an aqueous solution) and enzyme were mixed and then allowed to incubate overnight at 37°C in a shaking water bath. The buffer was either acetate (0.2 *M*, pH 5) for Suc d'*Helix pomatia* (20 μl of enzyme solution per ml of urine) or phosphate (0.5 *M*, pH 5) for the Serva enzyme (1500 units/ml of urine). The pH of the mixture was then adjusted to 6 by dropwise addition of 1 *M* sodium hydroxide. After the addition of dichloromethane (10 ml), the sample was extracted by shaking on a rotating shaker (10 min, 40 rpm) and then centrifuged (1000 *g*) at 10°C for 5 min. The upper aqueous layer was carefully aspirated and discarded. An aliquot of the organic phase (8 ml) was transferred into a new conical tube. Then 3 *M* hydrochloric acid (1 ml) was added and the mixture again shaken on a rotating shaker (10 min). After centrifuging (5 min), the organic phase was carefully aspirated and discarded. Then 3 *M* sodium hydroxide (1 ml) and 1 *M* phosphate buffer pH 6 (1 ml) were added to the remaining aqueous phase. The resulting aqueous solution (pH 6) was allowed to stand for 20 min and was then extracted with dichloromethane (10 ml). The aqueous phase was again aspirated and discarded. A 9-ml volume of the remaining organic phase was transferred into a new conical glass tube and evaporated to dryness under a gentle stream of nitrogen (35°C). The residue of the extract was redissolved in the eluent (300 μl) and injected for analysis (50 μl) (Fig. 2).

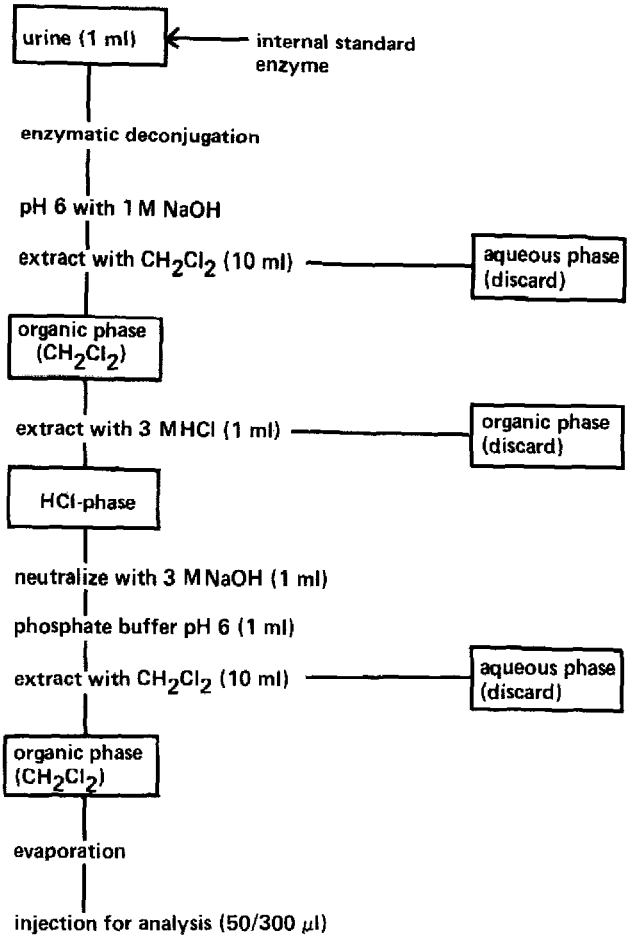


Fig. 2. Scheme for urine analysis.

Calibration

Plasma or urine standards (4—5) were processed as described above and analysed as calibration samples alongside the unknown samples. Peak height ratios of unchanged drug to internal standard were measured and the calibration curve was obtained from a least-squares linear regression. This regression line was then used to calculate the concentration of the drug in the unknown samples.

RESULTS

Recovery

Spiked plasma and urine standards of known concentrations were extracted as described above, except that the internal standard was not added. Another series of standards was prepared by adding solutions of I, II and III in the eluent to extracts of drug-free plasma (I) or urine (II, III). The peak heights obtained from this latter experiment provided the 100% values which could be compared with the peak heights obtained from the extracted spiked standards. The recovery of I from plasma was between 87% and 95% (Table I). The

TABLE I

RECOVERY OF BROMAZEPAM (I) FROM SPIKED PLASMA SAMPLES

Concentration (ng/ml)	Replicates (n)	Recovery (%)	C.V.* (%)
12.5	4	87	10.5
50.0	4	91	8.4
200	4	95	3.2

*C.V. = coefficient of variation.

TABLE II

RECOVERY OF 3-HYDROXYBROMAZEPAM (II) AND 3-HYDROXY-ABBP (III) FROM SPIKED URINE SAMPLES

Concentration (ng/ml)	Replicates (n)	II		III	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
250	4	53	2.8	49	10.8
400	4	62	4.8	51	7.6
1000	4	60	9.0	58	9.6
2000	4	56	4.6	59	5.4

recovery of II and III from urine was between 53% and 62%, and between 49% and 59%, respectively (Table II).

Linearity

A linear correlation between peak height ratios and the concentration of I in plasma or II and III in urine was found in the range of the calibration standards used (12.5—200 ng/ml in plasma, 250—2000 ng/ml in urine).

TABLE III

INTER-ASSAY PRECISION OF BROMAZEPAM (I) IN PLASMA

Experiment was performed with volunteer plasma samples.

Sample	Replicates (n)	Mean concentration found (ng/ml)	C.V. (%)
101	4	88	4.0
103	3	77	2.6
104	3	72	2.8
105	4	71	3.3
107	3	74	1.6
110	4	61	2.3
112	3	50	3.1
113	4	26	7.8
114	3	16	9.4
115	3	8	8.3

TABLE IV

INTER-ASSAY PRECISION OF 3-HYDROXYBROMAZEPAM (II) AND OF 3-HYDROXY-ABBP (III) IN URINE

Experiment was performed with volunteer urine samples.

II		III		*n*
Mean concentration found (ng/ml)	C.V. (%)	Mean concentration found (ng/ml)	C.V. (%)	
213	8.2	266	8.3	3
338	7.0	560	5.9	4
429	1.6	797	6.5	3
648	8.9	897	6.5	3
864	6.5	1170	3.9	3
1080	7.9	2090	6.8	3
1360	4.8	6330	8.2	3

Reproducibility

Inter-assay precision was calculated from volunteer plasma or urine samples, which were analysed as replicates on different days using a new calibration each day. For plasma samples, the mean coefficient of variation was found to be ± 2.8% (concentrations > 25 ng/ml) and ± 8.5% (concentrations < 25 ng/ml) (Table III). For urine samples, the mean coefficient of variation was found to be ± 6.4% (II) and 6.6% (III) (Table IV).

Detection limit

Under the conditions described, the limit of detection for I in plasma was

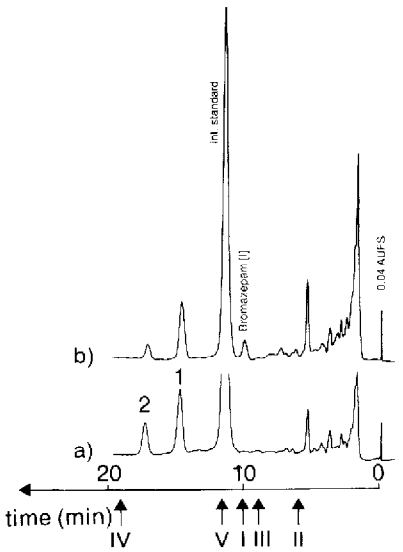


Fig. 3. Chromatograms of plasma extracts after a 6-mg oral dose of bromazepam to a volunteer. The arrows indicate the retention times of the compounds from Fig. 1. Peak 1 = impurity from plasma; peak 2 = impurity from the Extrelut material. (a) Pre-dose plasma, (b) plasma, 48 h after administration. Bromazepam concentration = 11 ng/ml.

6 ng/ml plasma. At this concentration a signal-to-noise ratio of 5:1 was observed. The detection limit for compounds II and III in urine was 200 ng/ml.

Application of the method

Plasma and urine samples from volunteers receiving 6 mg oral and intravenous administrations of bromazepam were analysed by this method. Fig. 3 shows chromatograms of plasma extracts from a volunteer. Plasma levels of the parent drug after oral administration of bromazepam to a volunteer are presented in Fig. 4. In this case the elimination half-life $t_{½\beta}$ was found to be 15 h. Fig. 5 shows chromatograms of urine extracts of a volunteer.

The absolute bioavailability of an oral solution of bromazepam is lower than that of the conventional formulation. As a possible explanation for this, ring opening of bromazepam at the low pH which obtains in the stomach, followed

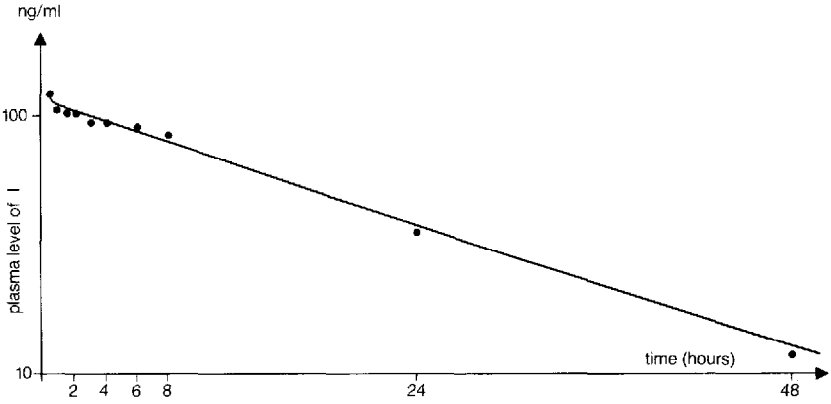


Fig. 4. Plasma levels of bromazepam after a 6-mg oral dose of bromazepam to a volunteer. Elimination half-life $t_{½\beta}$ = 15 h.

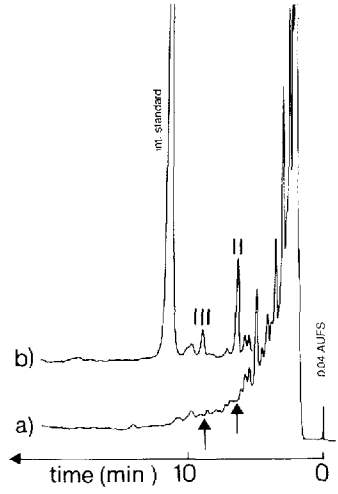


Fig. 5. Chromatograms of urine extracts after a 6-mg intravenous dose of bromazepam to a volunteer. Enzyme: β-glucuronidase from bovine liver, 3 h incubation. (a) Pre-dose urine, (b) urine fraction 12—24 h after administration. Concentration of II = 340 ng/ml, concentration of III = 150 ng/ml.

TABLE V

EXCRETION OF 3-HYDROXYBROMAZEPAM (II) AND OF 3-HYDROXY-ABBP (III) IN URINE FOLLOWING ORAL OR INTRAVENOUS APPLICATION OF 6 mg OF BROMAZEPAM TO A VOLUNTEER

Urine fraction (h)	II				III			
	Oral		Intravenous		Oral		Intravenous	
	ng/ml	Percentage dose cumulative	ng/ml	Percentage dose cumulative	ng/ml	Percentage dose cumulative	ng/ml	Percentage dose cumulative
0—12	200	2.1	614	9.8	1880	20.3	324	5.1
12—24	334	7.1	975	16.6	226	23.6	210	7.1
24—36	338	13.5	292	20.1	130	26.0	—	—

by formation of metabolite III from this open form, was invoked. This hypothesis was proved by determination of the corresponding metabolites in urine [8]. More of metabolite III than metabolite II was found following oral administration of bromazepam. After intravenous administration, more of metabolite II was found (Table V).

DISCUSSION

In our hands, the direct GLC measurement of underivatized bromazepam suffered from strong adsorption of bromazepam to the stationary phase. The two hydrolytic GLC assays for the determination of I in plasma were time-consuming and not specific, since metabolites II and IV, if present in plasma, would yield the same hydrolysis product. The present HPLC assay for the determination of bromazepam in plasma does not require any derivatization procedure or time-consuming clean-up step. The method is specific, since all known metabolites are separated from the parent drug.

Classical liquid—liquid extraction, using a 10-ml portion of dichloromethane, gave adequate recovery and clean extracts as did solid extraction columns. However, extraction with Extrelut 1 columns proved to be the most convenient and most rapid technique for this purpose. The columns were used without exit cannulae to avoid an interfering compound from this plastic material. Under the conditions described, the C_{18} material from Supelco was the most stable of those used by us.

Slight variations in the eluent composition had a marked influence on the retention times. For instance, by increasing the methanol content from 47% to 50% retention times were reduced for about 20%. Reducing the molarity of the buffer solution resulted in a poor peak shape for both I and V.

The determination of II and III in urine, following direct extraction, was not possible due to endogenous interferences. A clean-up step (back-extraction) was necessary.

The overnight incubation was carried out for practical reasons, though a 3-h incubation was sufficient for quantitative deconjugation [1, 3]. Incubation with the Serva enzyme resulted in a cleaner extract, and we therefore recommend the use of this enzyme or any other purified β-glucuronidase for this purpose.

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