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Sensitive determination of bromazepam in human tissues using capillary gas chromatography–mass spectrometry

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Abstract

A reliable and sensitive gas chromatographic–mass spectrometric method was devised to determine the levels of bromazepam in human tissues. Bromazepam was extracted from body tissues using a three-step solvent extraction procedure. N-Desmethyldiazepam served as the internal standard. Selected ion monitoring with m/z 317 for bromazepam and m/z 270 for internal standard was used for quantitation. Calibration curves in all body tissues were linear over the concentration range from 50–500 ng/g. The lower detection limit in body tissues was 2–5 ng/g and the absolute recovery in body tissues was 27.8–68.0%. This method was used to determine the levels of bromazepam in tissues of an autopsied individual who had been prescribed psychotropic drugs and who was found dead in a car.

Keywords: Bromazepam

1. Introduction

Bromazepam, a bromo derivative of 1,4-benzodiazepine [Fig. 1, 7-bromo-1,3-dihydro-5-(2-pyridinyl)-2*H*-1,4-benzodiazepin-2-one], is mainly prescribed as an anti-anxiety agent. Although it is relatively safe in therapeutic doses, an acute overdose can lead to death, especially in combination with alcohol or other drugs [1].

Several methods have been used to determine levels of bromazepam in plasma or urine, including gas chromatography (GC) [2–5], high-performance liquid chromatography (HPLC) [6–9], and liquid chromatography in combination with mass spectrometry (LC–MS) [10,11]. Gas chromatography–mass spectrometry (GC–MS) has apparently not been used to determine levels of this drug and we

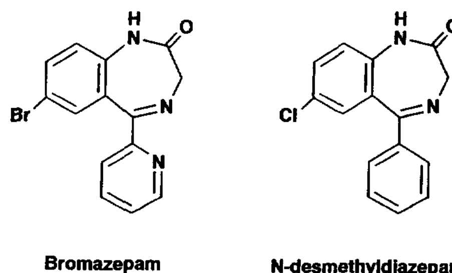

Fig. 1. Molecular structure of bromazepam and N-desmethyldiazepam (I.S.).

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found no documentation on determination of bromazepam in human solid tissues.

2. Experimental

2.1. Reagents

Bromazepam was provided by Nippon Roche K.K. (Tokyo, Japan) and N-desmethyldiazepam by Takeda Chemical Industries (Osaka, Japan). A carbonate buffer solution of pH 10.3 was prepared by mixing 0.1 *M* sodium carbonate solution and 0.1 *M* sodium bicarbonate solution (1:1). Ethyl acetate and *tert.*-butyl methyl ether were of analytical-reagent grade and were purified by distillation. Bromothymol blue indicator solution (0.04%) was purchased from Ishizu Seiyaku (Osaka, Japan). Other chemicals used were of analytical-reagent grade.

2.2. Biological samples

Human tissue samples were obtained at the time of autopsy and stored at −20°C until analysis. Drug-free human tissues were used as control samples.

2.3. Standard solution of bromazepam and internal standard (I.S.)

Bromazepam (5 mg) was dissolved in 5 ml of methanol to give a concentration of 1 μg/μl. This solution was further diluted to give concentrations of 100, 10 and 1 ng/μl. The standard solutions of I.S. (N-desmethyldiazepam) were prepared in the same manner.

2.4. Extraction procedure

The method of extracting bromazepam from human plasma, as reported by Solleu et al. [9] was modified so that solid tissue samples could be tested. An 0.5-g amount of tissue or 0.5 ml of whole blood was weighed and homogenized in 5 ml of carbonate buffer (pH 10.3) and 25 μl of I.S. solution (25 ng of N-desmethyldiazepam) in a 30-ml centrifuge tube. A 10-ml volume of *tert.*-butyl methyl ether was added and the preparation was shaken for 15 min and centrifuged at 850 *g* for 20 min. The solvent layer was transferred into a 30-ml centrifuge tube and a 2-ml volume of 2 *M* hydrochloric acid was added. The mixture was then shaken for 15 min and centrifuged at 850 *g* for 20 min. To remove lipids, the acid layer was washed with 5 ml of *tert.*-butyl methyl ether, in the same manner as above. The aqueous layer was transferred into a 10-ml centrifuge tube containing two drops of bromothymol blue solution (0.04%) as pH indicator and the mixture was made weakly alkaline (ca. pH 8) by adding 6 *M* and 0.1 *M* sodium hydroxide solution until the indicator turned pale blue. To the mixture were then added 1 ml of carbonate buffer (pH 10.3) and 5 ml of *tert.*-butyl methyl ether. The preparation was shaken for 15 min and centrifuged for 20 min. After centrifugation, the ether layer was dried with sodium sulfate for over 3 h and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 20 μl of ethyl acetate and a 2-μl aliquot of the solution was injected onto the gas chromatograph–mass spectrometer. The extraction procedure is summarized in Fig. 2.

2.5. Preparation of calibration curves

Samples (0.5 ml of whole blood or 0.5 g of tissue) were prepared to contain bromazepam at concentrations of 50–500 ng/ml or g, each containing 50 ng/ml or g of I.S. These samples were extracted in the same manner as described above. Calibration curves were obtained by plotting the peak-area ratios of bromazepam to I.S. versus the amounts of bromazepam.

2.6. GC–MS conditions

The apparatus used was a Hewlett-Packard 5971 mass selective detector with a Hewlett-Packard 5890 II gas chromatograph. The HP-1 fused-silica capillary column (13 m × 0.2 mm I.D., 0.33-μm film thickness) was coated with 100% dimethylpolysiloxane stationary phase. Splitless injection mode was selected with a valve off-time of 1 min.

The GC operating conditions were programmed at a rate of 30°C/min from 100°C to 250°C, the program was then slowed down to 20°C/min until 300°C. This temperature was maintained for 5 min.

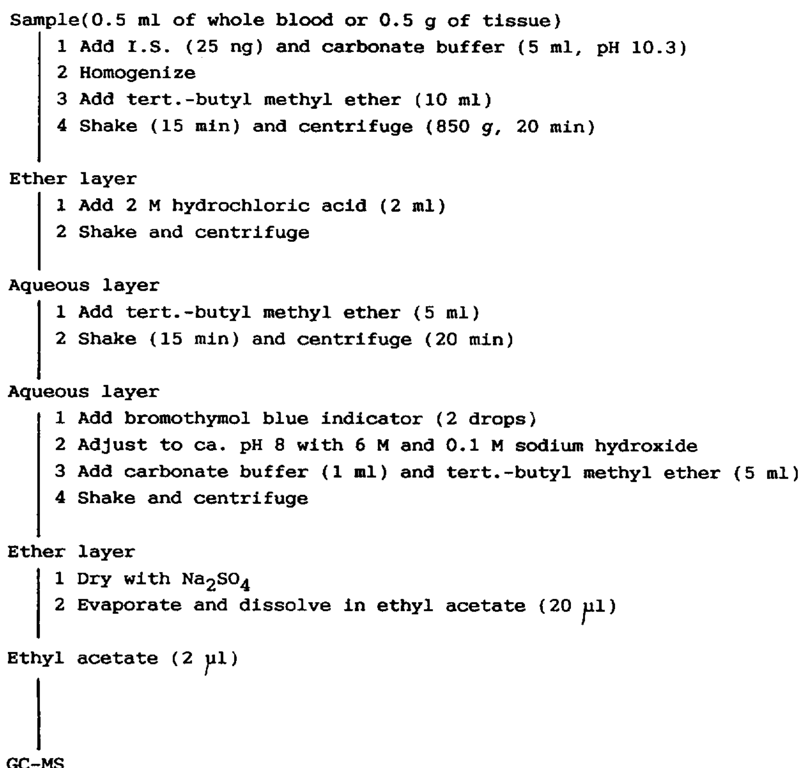

Fig. 2. Procedure for extracting bromazepam from human tissues.

Injection port and transfer line temperatures were 250°C and 280°C respectively. Helium was used as carrier gas with a column head pressure of 60 kPa (8.5 p.s.i.). The gas chromatograph–mass spectrometer was autotuned with perfluorotributylamine. The electron multiplier voltage was set 200 volts above tune value. For quantitation, two ions at m/z 317 ($[M+2]^+$) and 270 (M^+), were selected for bromazepam and I.S., respectively.

3. Results and discussion

3.1. Extraction procedure

Although a one-step extraction could give a higher recovery of the drug, many interfering peaks appeared on the gas chromatograms especially in body tissue samples and some extracts were yellow. This problem was overcome by back-extraction and selected ion monitoring in GC–MS.

As for the I.S., There was no proper I.S. available for the analysis of bromazepam, as the chromatographic property of bromazepam is different from that of other benzodiazepine compounds, except for N-desmethyldiazepam. As this compound is a benzodiazepine drug as well as a major metabolite of diazepam and chlordiazepoxide, there is the possibility that this compound is present together with bromazepam in the sample. We however chose this compound as I.S. since the deuterium-labeled N-desmethyldiazepam is commercially available in many countries, and can be used in such cases. N-desmethyldiazepam was extracted from tissues with a good recovery at pH from 9 to 14, but the recovery of bromazepam was extremely low at pH over 11. Therefore, a carbonate buffer of pH 10.3 was added after the neutralization of hydrochloric acid solution at the last step of extraction, and

bromazepam could be re-extracted without decomposition.

3.2. Determination of bromazepam in body tissues by GC–MS

Selected ion monitoring chromatograms of extracts from tissues containing 250 ng bromazepam and 25 ng I.S. are shown in Fig. 3. Each peak was clearly separated on the chromatograms, and significant interfering peaks were nil on the chromatograms of the drug-free human tissues (Fig. 4). The calibration curves for all tissues were linear in the concentration range from 50 to 500 ng/g. Correlation coefficients in all tissues exceeded 0.995. The slope of the calibration curves was slightly different for each tissue, and change depended on the degree of cleanliness of the glass insert at the injection port of the gas chromatograph. Therefore, use of the calibration curve obtained on the same day of analysis of practical samples is recommended.

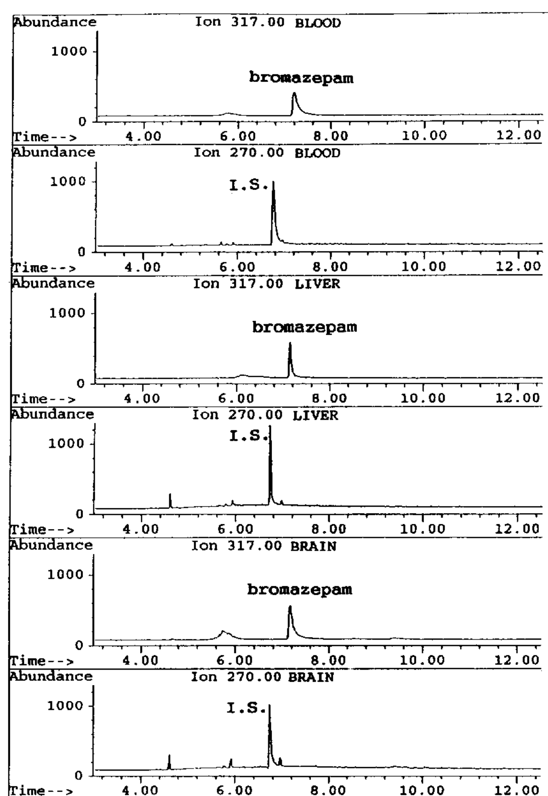

Fig. 3. Selected ion monitoring chromatograms of extracts from human whole blood, liver, and brain containing bromazepam (250 ng per sample) and I.S. (25 ng per sample).

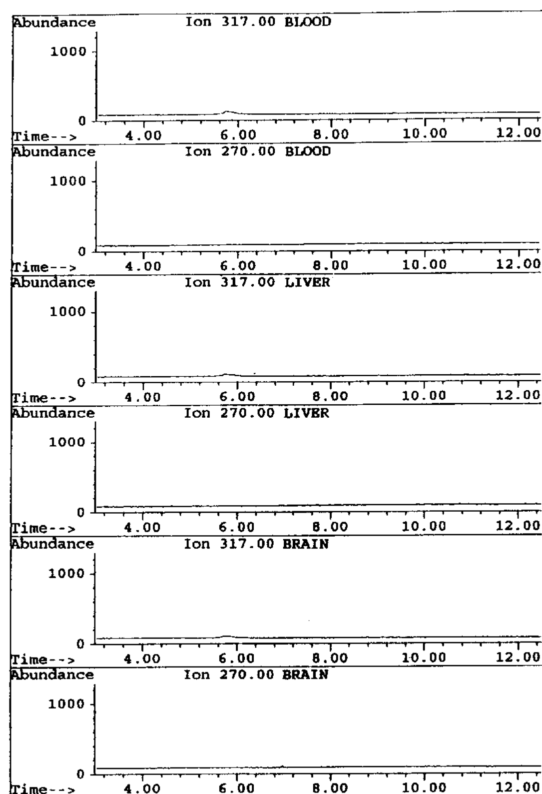

Fig. 4. Selected ion monitoring chromatograms of extracts from drug-free human whole blood, liver and brain.

The lower limit of detection with a signal to noise ratio 5:1 was 2–5 ng/ml (or g) in all samples examined, and could be lowered by injecting a more concentrated solution onto the gas chromatograph–mass spectrometer. The recovery of bromazepam in tissues was determined by adding 100 ng of bromazepam to 0.5 g of drug-free human tissue and then extracting in the same manner as for the sample. The absolute recovery calculated by comparing the peak area of bromazepam in tissue extracts with that of standard solution was 27.8–68.0%. Although the recovery of bromazepam in whole blood and liver was low, the limit of detection of 2–5 ng/g was considered to be sensitive enough to detect this drug at the therapeutic level, that is 107–173 ng/ml in whole blood [12].

Table 1
Precision and accuracy for analysis of bromazepam in tissues

Human tissue	Amount determined[a] (ng/g or ml)	C.V. (%)	Amount determined (ng/g or ml)	C.V. (%)
Whole blood	49.88±1.88	3.8	200.02±9.28	4.6
Brain	50.20±2.59	5.2	199.04±7.18	3.6
Lung	50.03±3.78	7.6	198.68±12.91	6.5
Kidney	49.82±2.61	5.2	198.56±11.73	5.9
Skeletal muscle	50.34±3.61	7.2	200.06±11.86	5.9
Liver	50.00±3.31	6.6	200.02±5.19	2.6
Spleen	49.82±2.77	5.6	200.88±6.24	3.1
Adipose	50.16±2.89	5.8	200.70±9.62	4.8

[a]Mean ± standard deviation.

The precision of this method for examining concentrations of 50 and 200 ng/ml or g in human tissues is summarized in Table 1 ($n=5$). The coefficients of variation ranged from 2.6 to 7.6%.

4. Practical application

A toxicological examination was made on a 71-year-old man who was found dead in a car in which exhaust gas had been introduced. Poisoning by carbon monoxide was ruled out as the CO-Hb level was only 10%. Toxicity related to a hospital administered prescription drug was suspected and general drug screening was carried out. As bromazepam was present in the whole blood along with several other psychotropic drugs, and no I.S. was detected in the sample, we determined concentrations of bromazepam in the tissues, using our method. Bromazepam was clearly present in all tissues examined, as shown in Table 2, but the whole blood level of this drug was considered to be therapeutic [12]. Therefore, toxicity by this drug in this patient was ruled out. The official cause of death was documented as poisoning by an overdose of psychotropic drugs including amitriptyline, triazolam and pentobarbital (data not shown).

Table 2
Bromazepam concentrations in autopsied human tissues

Sample	Concentration (ng/ml or g)
Whole blood	166.7
Liver	449.9
Kidney	363.0
Lung	202.4
Spleen	286.4
Adipose	147.0
Skeletal muscle	110.8
Brain	260.9

5. Conclusion

A reliable and sensitive method was devised to determine the levels of bromazepam in human solid tissues, using GC–MS. Our method made feasible forensic measurement of the distribution of bromazepam in human tissues.

Acknowledgments

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