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Note

Underivatized measurement of bromazepam by gas chromatography—electron-capture detection with application to single-dose pharmacokinetics

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Bromazepam, a 7-bromopyridinylbenzodiazepine derivative (Fig. 1) is extensively used in Europe and Canada for the treatment of anxiety and sleep disorders [1]. Several methods have been previously published for the measurement of bromazepam in plasma by high-performance liquid chromatography and gas chromatography [2–6]. The present method differs significantly from the other methods in its improved lower limit of sensitivity and ease of sample

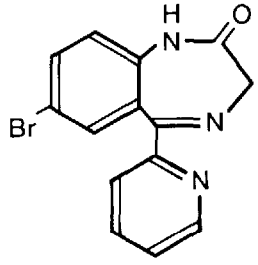

Fig. 1. Structural formula of bromazepam.

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preparation. In our laboratory the samples are autoinjected and 100 specimens can be analyzed in 24 h.

EXPERIMENTAL

Method

A Hewlett-Packard Model 5840A gas chromatograph equipped with a ^{63}Ni electron-capture detector and a 99-position automatic sampler was used. The column was coiled glass, 1.22 m × 4 mm I.D., packed with 10% OV-101 on 80—100 mesh Chromosorb W HP. The carrier gas was ultra-pure argon—methane (95:5) at a flow-rate of 55 ml/min. Operating temperatures were: injection port, 310°C; column, 275°C; detector, 310°C. New columns were conditioned at 290°C for 72 h with a carrier gas flow-rate of 10 ml/min. At the beginning of each assay run, the column was primed with 2 μg of phospholipid (asolectin) in benzene.

The following reagents were used as received from commercial sources: benzene, isoamyl alcohol, toluene and methanol.

Pure bromazepam (Ro 5-3350) and alprazolam (U-31889) (internal standard) were kindly provided by their manufacturers (Hoffmann-LaRoche and Upjohn, respectively). Bromazepam stock solution was prepared by dissolving 10 mg in 100 ml of toluene—methanol (90:10). Alprazolam stock solution was prepared by dissolving 10 mg in 100 ml toluene. Bromazepam and alprazolam stock solutions were then diluted 1:100 with toluene to yield 1 μg/ml working solutions. The working solutions were stored in the dark in glass-stoppered bottles at 4°C and were stable for at least one year. Stock solution in dark bottles at −20°C were stable for at least four years.

A 25-μl volume of alprazolam working solution (1 μg/ml), containing 25 ng alprazolam (internal standard), was added to a series of 15-ml round-bottom glass culture tubes with PTFE-lined screw-top caps. A 1-ml sample of unknown serum or plasma was added to each tube. Calibration standards for bromazepam were prepared by adding 5, 10, 25, 50, 75 and 100 ng of drug to consecutive tubes. Drug-free control serum or plasma was added to each of the calibration tubes. One blank sample, taken from the subject prior to drug administration, was analyzed with calibration standards and each set of unknown samples. A previously prepared and frozen serum quality-control sample of 25 ng/ml was thawed and analyzed during each sample run.

A 2-ml volume of benzene—isoamyl alcohol (98.5:1.5) was added to each tube and vortexed in the upright position for 2 min. The samples were then centrifuged at room temperature for 5 min at 400 *g*. The organic layer was transferred to 2-ml Wheaton automatic sampling vials and evaporated to dryness at 40°C at moderately reduced pressure. The samples were then reconstituted into 0.2 ml of toluene—isoamyl alcohol (85:15). A 6-μl volume was autoinjected and analyzed.

Single-dose pharmacokinetic study

A 60-year-old male weighing 75 kg and in good health participated after giving written informed consent. He received a single 6-mg oral dose of bromazepam following an overnight fast. Venous blood samples were drawn

into additive-free tubes prior to and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36 and 48 h post-dose. Serum was separated and frozen at − 20° C until the time of assay.

Pharmacokinetic analysis

The slope (β) of the terminal log linear phase of the serum concentration curve was determined by linear regression analysis. This was used to calculate the apparent elimination half-life. Area under the serum concentration curve (AUC) up to 48 h after dosage was determined by the trapezoidal method. To this was added the residual area extrapolated to infinity, calculated as the 48-h concentration divided by β, yielding the total AUC. Apparent oral clearance of bromazepam was calculated as the administered dose (6 mg) divided by AUC, assuming complete absorption.

RESULTS

Evaluation of the method

Under the described conditions the retention time for bromazepam was 3.85 min and for alprazolam 7.50 min (Fig. 2). The blank plasma or serum after extraction contained no interfering peaks. The relation between bromazepam concentrations and peak-height ratio (versus internal standard) was linear from at least 5 to 100 ng/ml. Correlation coefficients were always greater than 0.99. Relative standard deviation (standard deviation divided by mean expressed in %) calculated by analyzing six replicate known standards on the same day (1, 5, 10, 25, 50 and 100 ng/ml) were: 12.0, 5.0, 2.9, 2.9, 2.6 and 2.8%, respectively. The mean value of the 25 ng/ml quality-control standard

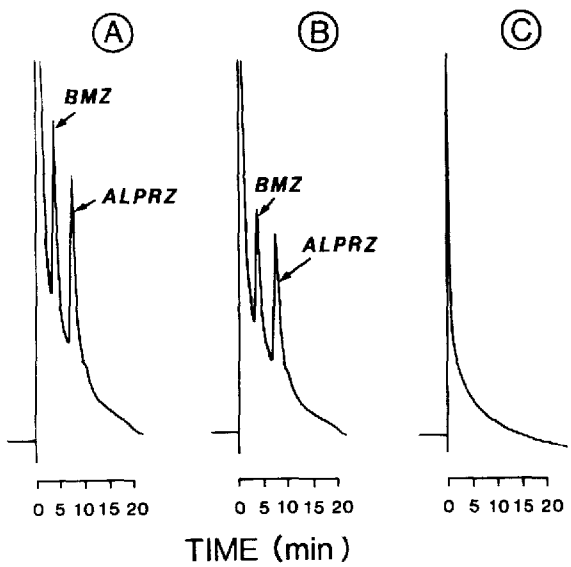

Fig. 2. (A) Chromatogram of a calibration standard containing 25 ng/ml bromazepam (BMZ) and 25 ng/ml alprazolam (ALPRZ), the internal standard. (B) Chromatogram of a patient sample 12 h following oral administration of 6 mg of bromazepam, containing 28.6 ng/ml. (C) Chromatogram of a drug-free blank sample.

across eight analytic runs was 25.8, with a between-day coefficient of variation of 7.4%.

Pharmacokinetic results

Fig. 3 shows serum bromazepam concentrations from the pharmacokinetic study. Kinetic variables were: peak serum concentration, 66 ng/ml; time of peak, 1.5 h post-dose; elimination half-life, 25.0 h; oral clearance, 78.1 ml/min.

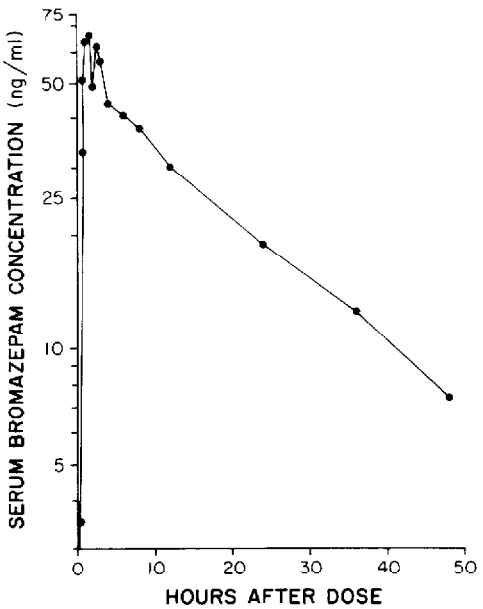

Fig. 3. Serum bromazepam concentrations following oral administration of bromazepam in the volunteer subject.

DISCUSSION

This report describes a reliable and specific method for the quantitation of underivatized bromazepam in plasma using gas chromatography with electron-capture detection. This method has improved sensitivity and greater simplicity of sample preparation compared with other previously published techniques for bromazepam [2—6]. Sensitivity is sufficient to carry out single-dose pharmacokinetic studies, using therapeutic doses. The method employs neutral benzene extraction from plasma and concentration into a small volume for autoinjection into the chromatograph equipped with an electron-capture detector. This method consistently provides blank plasma samples free of contaminants in the areas corresponding to the retention times for bromazepam and alprazolam (the internal standard).

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