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GAS CHROMATOGRAPHIC DETERMINATION OF BROMO AND FLUORO DERIVATIVES OF BENZODIAZEPINE IN HUMAN BODY FLUIDS

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SUMMARY

A rapid, sensitive and accurate method for the determination of bromazepam and flunitrazepam in plasma and urine using gas chromatography has been developed. Bromazepam was extracted with diethyl ether and flunitrazepam with hexane at pH 7. A nitrogen detector was used to determine bromazepam and an electron-capture detector was used for flunitrazepam.

INTRODUCTION

Bromazepam, a bromo derivative of benzodiazepine, is an anti-anxiety and hypnotic drug administered in therapeutic doses of 3—18 mg daily.

Flunitrazepam, a fluoro derivative of benzodiazepine, is an anaesthetic drug given in the dose range of 0.01—0.03 mg/kg. Such low therapeutic doses are connected with the strong action of these drugs and result in low concentrations in plasma and in urine. Literature reports describe the determination of these compounds by differential polarographic [1], spectrofluorodensitometric [2] and gas chromatographic [3, 4] methods. In the latter case benzophenones, formed as a result of hydrolysis of the mentioned compounds, were determined. Columns with different packings were used with an electron-capture detector (ECD). The hydrolysis of these compounds significantly prolongs the determination time.

Vree et al. [5] determined flunitrazepam quantitatively by high-performance liquid chromatography. Kaniewska [6], studying bromo and fluoro derivatives of benzodiazepine by gas chromatography, elaborated a sensitive identification and determination method for these compounds omitting the hydrolysis process.

Differential polarographic and spectrofluorodensitometric methods used for

determination of compounds in human body fluids are not sufficiently sensitive when therapeutic doses are administered.

It was the aim of this work to optimize the extraction conditions of bromazepam and flunitrazepam from human plasma and urine and then to determine them directly by gas chromatography [6]. The method was developed to facilitate pharmacokinetic investigations of the drugs.

EXPERIMENTAL

Materials and reagents

Bromazepam (Lexotan®) was obtained from Hoffmann-La Roche (Nutley, N.J., U.S.A.), the purity, determined by a titration method was 99.7%; flunitrazepam (Rohypnol®) was from Hoffman-La Roche, m.p. 167—168° and the purity determined by a titration method was 99.93%.

Apparatus

A Pye 104 model 84 gas chromatograph, a Perkin-Elmer Model 3903 gas chromatograph, and a Mechanika Precyzyjna centrifuge, type 317a were used.

Determination of bromazepam in human plasma

Determinations of bromazepam in human plasma were performed on a Perkin-Elmer Model 3903 chromatograph equipped with a nitrogen detector. A glass column (2 m × 0.3 cm I.D.), packed with 10% UCC-W-98 on Chromosorb W AW DMCS (80—100 mesh) was used. The column was conditioned at 240° for 4 h with no gas flow; then at 280° for 48 h. Operating temperatures were column 265°, injection port 275°, detector 275°. Nitrogen was used as the carrier gas at a flow-rate of 45 ml/min; the hydrogen flow-rate was 9.2 ml/min and the air flow-rate 150 ml/min. The sensitivity was set at 1 × 16. Diazepam was used as an internal standard in the quantitative analysis.

Standard solutions. Two stock solutions were made up: (1) 100 mg bromazepam in 100 ml ethanol and (2) 50 mg diazepam in 100 ml ethanol. From the stock solutions, four standard solutions were prepared containing 2.5, 5, 7 and 10 μg of bromazepam per 100 μl.

Preparation of calibration curves. From the standard solutions calibration curves were constructed omitting the extraction process (curve I) and after an extraction from human plasma (curve II). In the latter case to each of the four centrifugal tubes 1 ml of human plasma and 100 μl of the standard bromazepam solutions were added and stirred. The mixtures were extracted with 4 ml diethyl ether three times, shaking each time for 15 min. After centrifugation for 5 min at 4000 *g* the upper organic layer was removed using a pipette, transferred to a conical centrifuge tube and dried with anhydrous sodium sulphate. After filtration the diethyl ether was evaporated under vacuum below 30°. The residue was dissolved in 0.2 ml of the internal standard solution (10 μg/ml). To prevent solvent evaporation the tubes were cooled in iced water. An aliquot of 2 μl of the final solution was injected onto the chromatographic column.

The linearity of the detector was determined by the calibration curves. Calibration curves I and II expressed the ratio dependence of the bromazepam

peak of that of internal standard on the concentration of bromazepam. From the curves thus obtained the recovery of bromazepam from human plasma was determined.

The content of bromazepam may be determined in 1—4 ml of human plasma under the same extraction conditions as those for the preparation of calibration curve II.

The concentration of bromazepam was calculated from calibration curve II prepared separately for each series of determinations.

Determination of bromazepam in human urine

Preparation of calibration curves. To each of four centrifuge tubes 1 ml of urine was added and adjusted to pH 7.0 with borate buffer, pH 9.0. Then 100 µl of the ethanol standard solutions containing 2.5, 5, 7.5, and 10 µg of bromazepam were added. Extraction and determination processes were performed as described for the determination of bromazepam in plasma.

From the standard solutions calibration curve I was constructed directly, i.e. omitting the extraction process; calibration curve III was prepared after the extraction from urine (Fig. 1).

For determination of unknown aliquots of bromazepam 1—4 ml of urine was used and the extraction conditions were the same as those given for the standard curves. The concentration of bromazepam was determined from calibration curve III which was prepared separately for each series of determinations.

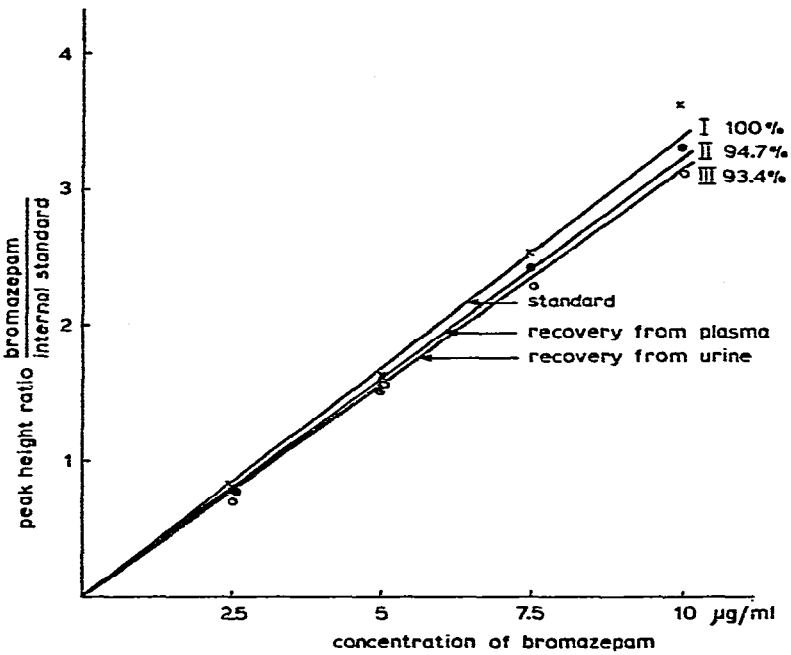

Fig. 1. Calibration curves of bromazepam: I, (x) omitting the extraction process; II (•) after extraction from plasma; III, (o) after extraction from urine.

Determination of flunitrazepam in human plasma

Determination of flunitrazepam in human plasma was performed on a Pye 104 gas chromatograph equipped with an ECD by the method developed previously [6]; a glass column (1.65 m × 0.4 cm I.D.) packed with 10% UCC-W-98 on Chromosorb W AW DMCS (80—100 mesh) was used. Operating temperatures were: Column 260°, injection port, 290°, and detector, 300°. Argon was used as the carrier gas at a flow-rate of 60 ml/min, the hydrogen flow-rate was 45 ml/min and the air flow-rate 300 ml/min. The sensitivity was set at 10×10^2. Diazepam was used as an internal standard in the quantitative analysis.

Standard solutions. Two stock solutions were made up: (1) 5 mg flunitrazepam in 100 ml ethanol and (2) 10 mg diazepam (internal standard) in 100 ml ethanol.

From the stock solutions three standard solutions were prepared containing 12.5, 25 and 50 ng of flunitrazepam per 100 µl.

The extraction process and determinations were as indicated for bromazepam except that hexane was used and the internal standard concentration amounted to 500 ng/ml.

From the standard solutions calibration curve I was construced directly, i.e. omitting the extraction process; calibration curve II was prepared after extraction from plasma.

The curves obtained were then used for calculation of the recovery and verification of the detector linearity in the concentration range 12.5—50 ng/ml (Fig. 2).

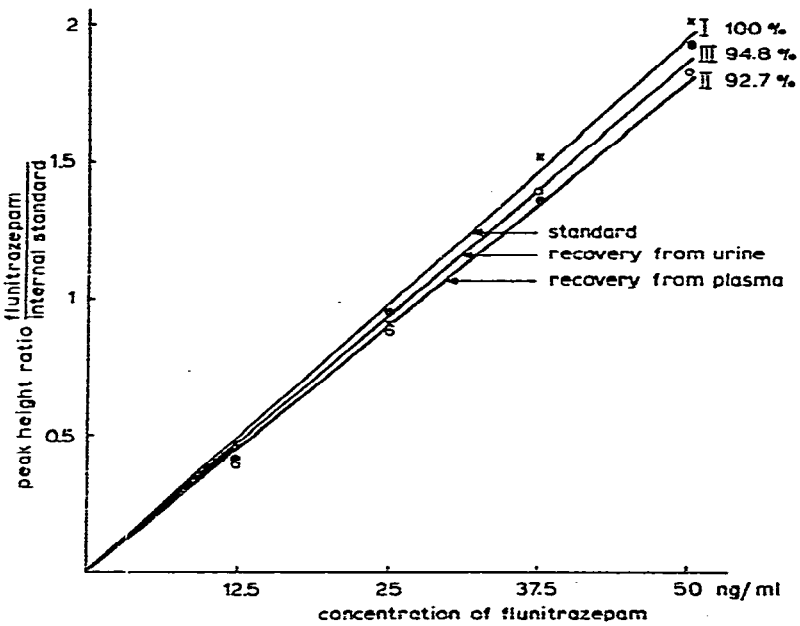

Fig. 2. Calibration curves of flunitrazepam: I, (x) omitting the extraction process; II, (o) after extraction from plasma; III (•) after extraction from urine.

For determination of unknown aliquots of flunitrazepam 1—4 ml of plasma was used and the extraction conditions were the same as those for the preparation of the calibration curve for nitrazepam.

The concentration of flunitrazepam was determined from calibration curve II prepared spearately for each determination series.

Determination of flunitrazepam in human urine

Preparation of calibration curves. To each of three centrifuge tubes 1 ml of urine was added and adjusted to pH 7.0 with borate buffer, pH 9.0. Then 100 µl of the standard solutions of flunitrazepam (12.5, 25, 50 ng) were added and extracted three times with 4 ml of hexane, shaking each time for 15 min.

After centrifugation (5 min at 4000 *g*) the upper organic layer was transferred to a conical centrifuge tube and dried with anhydrous sodium sulphate. After filtration the hexane was evaporated under vacuum at 40°. The residue was dissolved in 0.2 ml of internal standard solution (500 ng/ml).

Aliquots of 2 µl of each of the final solutions were injected on the chromato-

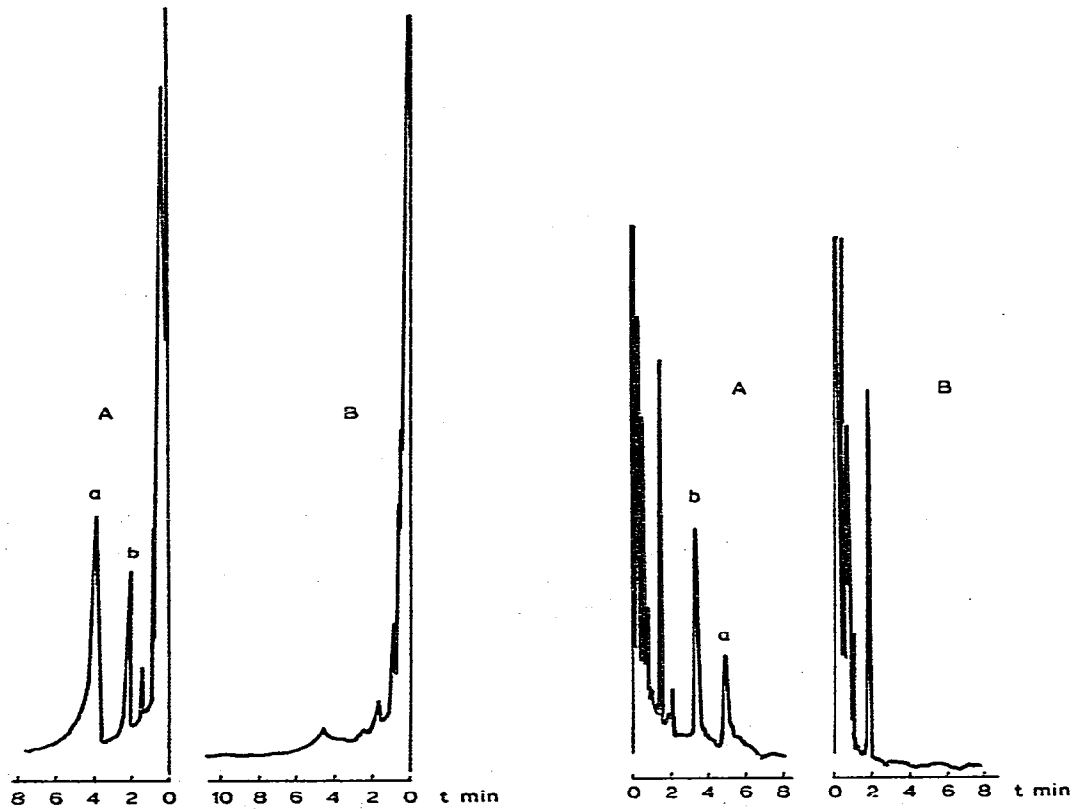

Fig. 3. Gas chromatograms of the extract from plasma containing bromazepam and diazepam (A) and of the extract from blank plasma (B). Peaks : (a) bromazepam, (b) diazepam (internal standard).

Fig. 4. Gas chromatograms of the extract from plasma containing flunitrazepam and diazepam (A) and of the extract from blank plasma (B). Peaks: (a) flunitrazepam, (b) diazepam (internal standard).

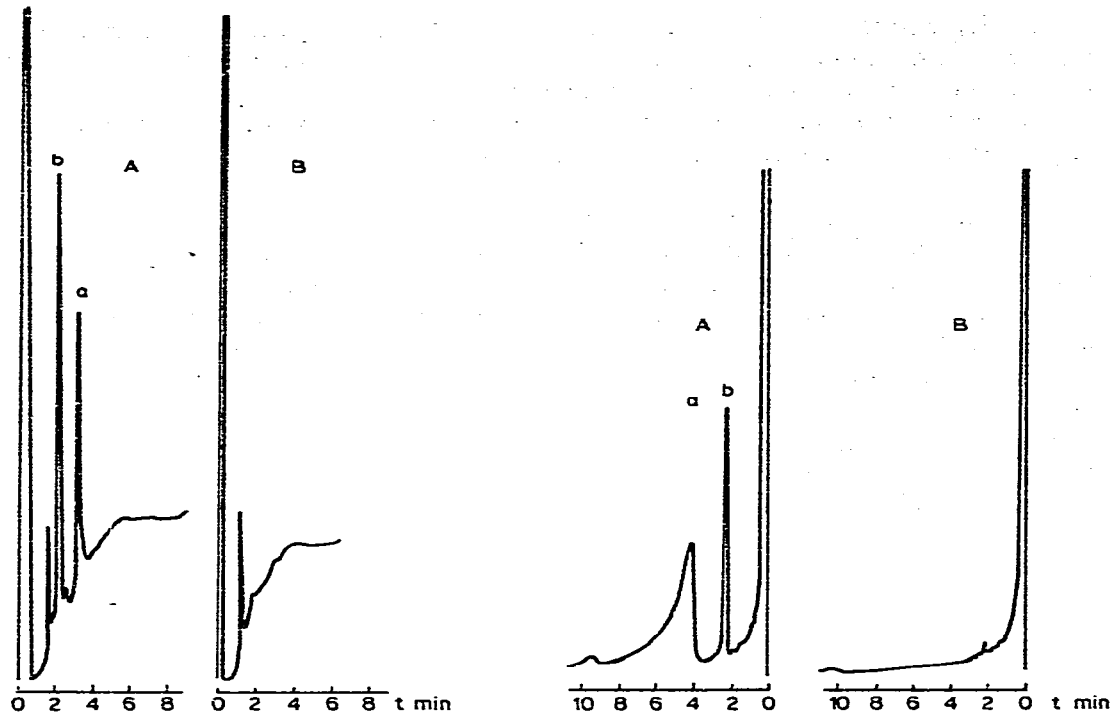

Fig. 5. Gas chromatograms of the extract from urine containing flunitrazepam and diazepam (A) and of the extract from blank urine (B). Peaks: (a) flunitrazepam, (b) diazepam (internal standard).

Fig. 6. Gas chromatograms of the extract from urine containing bromazepam and diazepam (A) and of the extract from blank urine (B). Peaks: (a) bromazepam, (b) diazepam (internal standard).

graphic column and the calibration curve was prepared. At the same time the calibration curve of flunitrazepam was constructed omitting the extraction process (Fig. 2).

For the determination of unknown concentrations of flunitrazepam 1—4 ml of urine was used and the extraction conditions were the same as those given for the standard curves. The concentration of flunitrazepam was determined from calibration curve III prepared separately for each series of determinations.

RESULTS AND DISCUSSION

The use of diethyl ether and hexane for the extraction of bromazepam and flunitrazepam from plasma and urine was convenient because of their relatively low specific weights. The organic layer accumulated at the top and was easy to separate. The extraction process was optimal at pH 7.0, thus requiring the urine to be adjusted with buffer to pH 7.0.

Fig. 3 shows chromatograms of plasma with added bromazepam and diazepam (internal standard), and blank plasma. Fig. 4 shows chromatograms of plasma with added flunitrazepam and diazepam, and blank plasma. Fig. 5 shows chromatograms of urine with added flunitrazepam and diazepam, and

blank urine. Fig. 6 shows chromatograms of urine with added bromazepam and diazepam, and blank urine.

For detecting bromazepam by gas chromatography a nitrogen detector was used because despite the fact that this compound contains a halogen in the molecule it gave poor peak shape (broad tailing peaks) and poor ECD response.

This allows the determination of bromazepam in plasma and urine in the concentration ranges 2.5—10 μg/ml where the recovery of bromazepam from plasma amounted to 94.7 ± 7.5% and from urine to 93.4 ± 8% (n = 10). The sensitivity limit of the method for this compound was 0.6 μg/ml.

Statistical evaluation for the extraction of bromazepam from plasma is as follows: $\bar{x}$ = 96.65%, s = 3.36, confidence interval 96.65% ± 3.52 for p = 0.95, n = 7, C.V. = 3.24%. For extraction from urine $\bar{x}$ = 95.33%, s = 4.23, confidence interval 95.33% ± 3.25 for p = 0.95%, n = 9, C.V. = 4.44%.

The use of the ECD which is sensitive to halogens allowed the determination of flunitrazepam in plasma and urine in the concentration range 12.5—50 ng/ml. The sensitivity limit of the method for this compound was 3 ng/ml in plasma and urine. In the given concentration ranges of flunitrazepam in plasma the average recovery was 92.7 ± 8% (n = 15) and 94.8 ± 6.5% (n = 6) in urine.

Statistical data for the extraction of flunitrazepam from plasma are as follows: $\bar{x}$ = 97.25%, s = 4.47, confidence interval 97.25% ± 5.11 for p = 0.95, n = 7, C.V. = 4.59%. For the extraction from urine $\bar{x}$ = 98.61%, s = 3.69, confidence interval 98.6% ± 4.24 for p = 0.95, n = 6, C.V. = 3.81%.

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