Journal of Chromatography, 222 (1981) 501—506
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 764

Note

Determination of bromazepam by gas—liquid chromatography and its application for pharmacokinetic studies in man

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(First received August 18th, 1980; revised manuscript received October 24th, 1980)

Bromazepam is a relatively new drug of the well known 1,4-benzodiazepine class of anxiolytic compounds. It is increasingly used and knowledge of its pharmacokinetic properties may contribute to a safe and rational therapy. This necessitates a specific and sensitive measurement in biological fluids. In the past different methodological approaches have been used, such as direct gas—liquid chromatographic (GLC) measurements of unchanged substance [1] or as hydrolyzed 2-amino-5-bromobenzoylpyridine (ABBP) [2, 3] as well as thin-layer chromatography either directly or after derivatization of bromazepam to an azo-dye on the plate [4]. Apparently, all three methods suffer from different pitfalls and limitations (see Discussion). Because bromazepam is avidly absorbed onto the stationary phases of gas chromatographic column packings, we developed a new assay based on the methylation of bromazepam to its N^1-methyl derivative, resulting in a marked improvement of its GLC performance. The method can easily be applied to monitoring plasma levels in patients treated with bromazepam.

EXPERIMENTAL

Chemicals

Diazepam, bromazepam and N^1-methylbromazepam were kindly donated by Hoffmann-La Roche (Grenzach, G.F.R.). Diethyl ether (nanograde) was purchased from Mallinckrodt (Promochem, Wesel, G.F.R.); iodomethane and tetrabutylammonium iodide were from Fluka (Buchs, Switzerland).

Apparatus

A gas chromatograph Varian 2700 equipped with a ^{3}H-Sc-ECD was used and coupled to a 1-mV data recorder (chart paper speed 0.5 cm/min).

0378-4347/81/0000—0000/$02.50

GLC conditions

Purified nitrogen, at a flow-rate of 40 ml/min, was used as carrier gas. The injection port and the electron-capture detector (ECD) were maintained at 320°C. The temperature of the 1-m coiled glass column filled with 0.5% OV-17 Chromosorb G HP 100—200 mesh (Applied Science Labs., State College, PA, U.S.A.) was 270°C. The new column was conditioned once with Silyl-8 (Pierce, Rockford, IL, U.S.A.) and every working day about three injections with a test solution containing diazepam and methylbromazepam were carried out.

Sample preparation

Stock solutions of diazepam (100 ng/ml) and bromazepam (500 ng/ml) were made with benzene. To 0.5—1 ml plasma 10 ng diazepam (internal standard) and 2 ml 0.1 *N* sodium hydroxide were added. A calibration curve between 10 and 100 ng bromazepam per ml was run with blank plasma. Plasma samples were extracted twice with 6 ml diethyl ether. The combined organic phases were concentrated under nitrogen to about 4 ml. After addition of 100 μl iodomethane and 250 μl tetrabutylammonium iodide (0.025 *M* in 0.1 *N* sodium hydroxide) samples were shaken for 1 h at room temperature. Following this methylation reaction the separated ether phase was extracted with 3 ml 1 *N* hydrochloric acid. After centrifugation the upper organic layer was discarded, the aqueous phase alkalinized with 1.5 ml 2.5 *N* sodium hydroxide and back extracted into diethyl ether (7 ml). This separated and purified ether extract was vaporized in silanized glasses and the residue was redissolved in 20 μl of benzene. A 2-μl aliquot of this solution was injected into the gas chromatograph.

Assignment of the peaks

Qualitative peak assignment was carried out in two ways: first, by comparing the retention times of the peaks to those of known reference compounds and secondly, by comparing the mass spectra of the different peaks. Quantitative assignment was performed by calculating the ratios of the peak heights of the drug to that of the internal standard and relating this to a concomitantly constructed linear calibration curve over the concentration range of 10—100 ng/ml.

RESULTS

Many different liquid phases and various procedures for column conditioning were tried to perform the direct measurement of bromazepam according to De Silva et al. [1]. However, due to the strong absorption of this compound broad and excessively tailing peaks were observed (Fig. 1a). Multiple injections of identical standard solutions as well as of biological samples (after different extraction procedures) resulted in a very wide variation of peak height and areas. Besides the very poor reproducibility, sensivity was also bad (lower limit varying between 50 and 100 ng/ml).

The methylation of bromazepam to its N^1-methyl derivative resulted in a significant improvement of the GLC measurements (Fig. 1c). Multiple direct injections of standard solutions of methylbromazepam and diazepam revealed

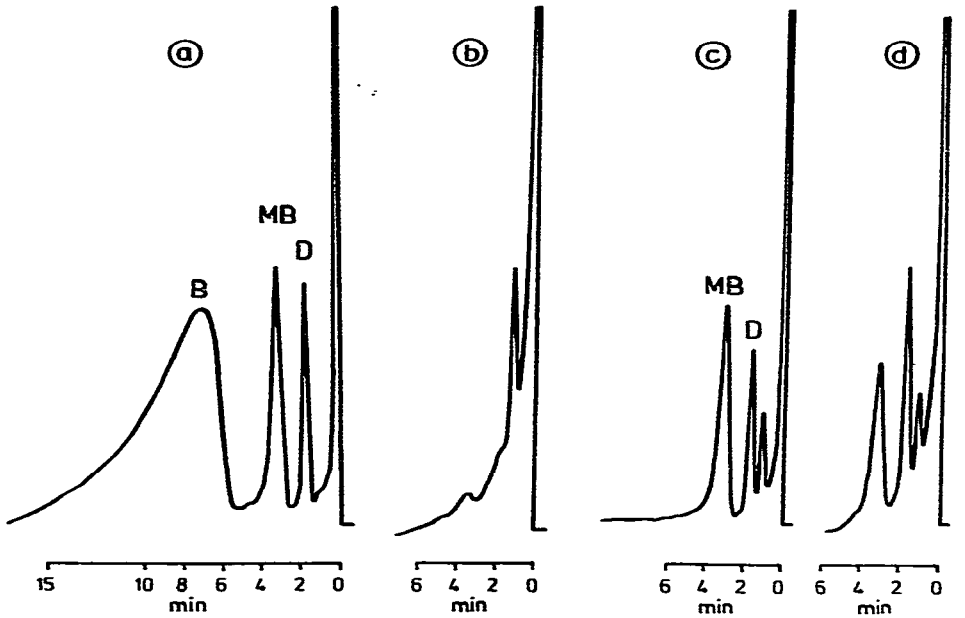

Fig. 1. (a) Chromatogram of standard solutions of the internal standard diazepam (D), N^1-methylbromazepam (MB) and bromazepam (B); concentration of B 25 times higher than that of MB. (b) Chromatogram of an extracted and derivatized control (blank) plasma (two-fold amplified in comparison to a, c and d). (c) Chromatogram of an extracted and derivatized standard plasma spiked with bromazepam and diazepam. (d) Chromatogram of an extracted and derivatized patient's sample.

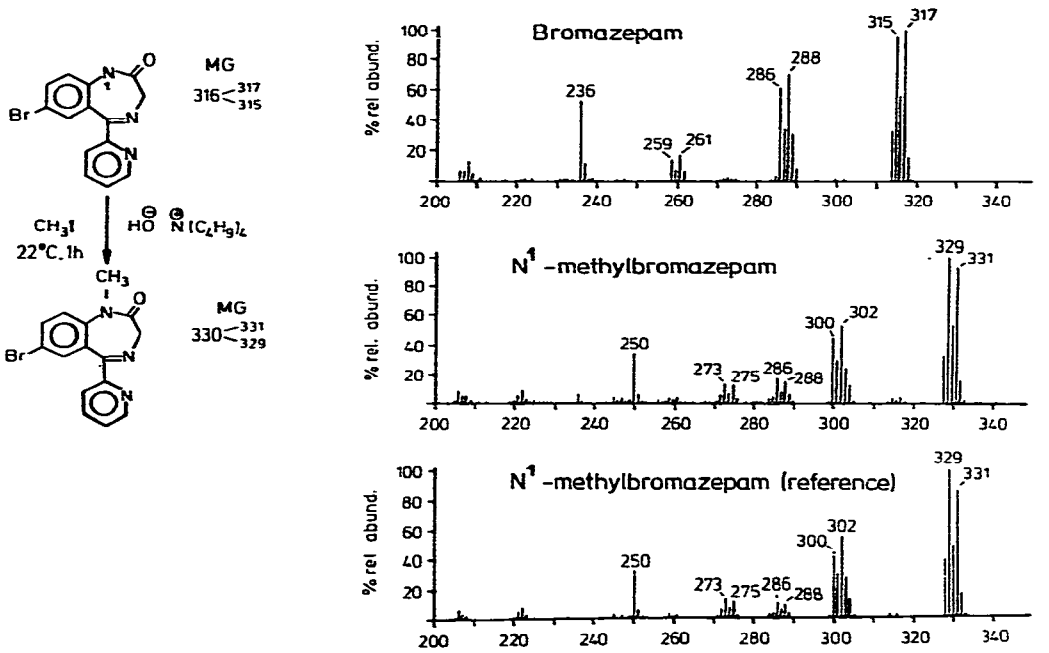

Fig. 2. Mass spectra and formulae of bromazepam (top) and its N^1-methyl derivative (middle), in addition the conditions for the methylation reaction are given (left); for comparison the mass spectrum of an authentic sample of N^1-methylbromazepam (Ro 5-4547) is shown below.

that the variation between different injections was only 3% indicating stabile GLC performance. After the complete extraction and derivatization procedure, recovery averaged 60% and multiple daily analyses (n=10) of a biological quality control sample containing 50 ng/ml revealed a value of 49.8±3.4 (mean ±S.D.).

The GLC peak pattern as well as analysis of the mass spectra proved that the derivatization reaction resulted in a quantitative and specific methylation of the nitrogen in position 1 (Fig. 2). The extensive clean-up procedure was primarily necessary to separate peaks with retention times shorter than that of diazepam. Thereby the samples could be measured at a higher amplifier setting and sensitivity was increased. After the complete sample preparation diazepam and methylbromazepam gave well-shaped, symmetrical peaks with retention times of 1.8 and 3.2 min, respectively. Thus every 5 min a sample could be injected (see Fig. 1). No interfering peaks could be observed in samples of patients not receiving bromazepam (see Fig. 1b). 3-Hydroxybromazepam (major metabolite in plasma) and the benzoylpyridine derivative (major urinary metabolite; 1) have been separated by the multiple steps of derivatization and extraction.

Calibration curves were linear over the concentration range tested (10—100 ng/ml) and the lower limit of sensitivity (5 ng/ml plasma) allows the measure-

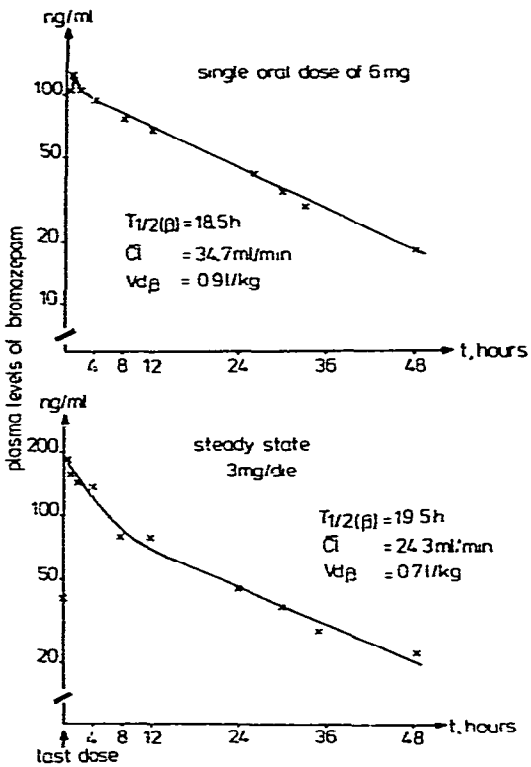

Fig. 3. Plasma concentration—time profiles of bromazepam in the same subject following a single oral dose of 6 mg/day (top) and multiple dosing with 3 mg/day (bottom). The arrow indicates the time of the fifth oral dose of bromazepam. $T_{½(\beta)}$ = elimination half-life; $\overline{Cl}$ = total body plasma clearance; Vd_β = apparent volume of distribution.

ment of clinically relevant plasma levels of bromazepam. This can be directly seen from our pharmacokinetic studies. Following a single therapeutic dose of 6 mg bromazepam (Lexotanil®) or the multiple oral dosing with 3 mg per day, plasma concentrations were followed for 48 h in healthy volunteers (Fig. 3). Thus, the new method can be applied for the calculation of all important pharmacokinetic parameters either from single-dose studies or under steady state conditions.

DISCUSSION

Several methods have been published so far for the quantitative analyses of the anxiolytic compound bromazepam [1—4]. However, all assays apparently seem to have their limitations. The direct measurement of bromazepam by GLC—ECD according to De Silva et al. [1] did not give, at least in our hands, satisfactory results. Due to the strong absorption of bromazepam to a variety of stationary phases tested no consistent and reproducible results could be obtained. This problem could not be solved by injecting control blood extracts as suggested by De Silva et al. Their method was also complicated by absorption phenomena since they had to make "10-μl injections of the external standards between every two or three consecutive biological samples". Thus, it appears that the application of this method is not suitable for routine use.

Another approach was the acid hydrolysis of bromazepam to ABBP [2, 3]. This method is not specific for bromazepam since two metabolites of bromazepam yield the same hydrolysis product [1]. This interference could be particularly troublesome after multiple dosing with bromazepam when these metabolites might accumulate in plasma.

More recently Haefelfinger [4] reported a method with thin-layer plates. The direct measurement of the UV reflectance of bromazepam or the colorimetric measurement of ABBP preceded by an extraction procedure does not include an internal standard and the assay shows interferences by unknown plasma constituents and probably by other drugs. Therefore its use in clinical practice might be limited.

Consequently a specific, sensitive and more practicable assay needed to be developed. From our experience with the ECD—GLC measurements of diazepam and its demethylated major metabolite N-desmethyldiazepam [5] we assumed that loss of the nitrogen in position 1 is associated with increasing absorption to the column filling. Therefore derivatization of bromazepam to its N^1-methyl analogue was performed. Such methylation has been successfully applied to the analysis of clonazepam and nitrazepam [6]. As can be seen from Figs. 1 and 2 methylation of bromazepam to N^1-methylbromazepam was specific and quantitative. In contrast to the parent drug the reaction product was not absorbed on the stationary phase and yielded reproducible results with high specificity and sufficient sensitivity. The presented method can be performed by any laboratory equipped with a GLC—ECD system and about 20 samples can be run during one day. From our application to pharmacokinetic questions it can be concluded that the assay can be used also for routine plasma level monitoring. Some important pharmacokinetic parameters have been calculated from the plasma concentration—time profiles (see Fig. 3) and the presented values

are in agreement to the limited literature data [7, 8]. Recently a direct GLC assay for bromazepam was reported [9]; however, the lower limit of sensitivity (600 ng/ml) considerably exceeds the maximal plasma levels (between 50 and 230 ng/ml) observed in man. Thus, this method can not be applied to pharmacokinetic investigations as suggested by the authors.

In summary, this new assay has overcome the difficulties and limitations of published methods and seems to be a method of choice for the determination of bromazepam in human biological fluids.

ACKNOWLEDGEMENTS

This study was supported by the Robert Bosch Foundation, Stuttgart, G.F.R. The author is greatly indebted to Dr. C. Fischer for the evaluation of the mass-spectral data and to Miss Kopp for her helpful technical assistance.

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