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DETERMINATION OF THE NEW MONOAMINE OXIDASE INHIBITOR BROFAROMINE AND ITS MAJOR METABOLITE IN BIOLOGICAL MATERIAL BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A sensitive gas chromatographic assay for the simultaneous determination of brofaromine [4-(7-bromo-5-methoxy-2-benzofuranyl)piperidine hydrochloride], a new monoamine oxidase-A inhibitor, and its major metabolite was developed and validated. After addition of 4-(5-bromo-2-benzofuranyl)piperidine as internal standard, the compounds were isolated from biological fluids by liquid–liquid extraction at basic pH. After derivatization with heptafluorobutyric anhydride the compounds were chromatographed using a packed column (OV-17) and an electron-capture detector. The limit of quantitation was ca. 0.03 nmol per sample (10 ng) for both compounds. Analysis of spiked samples demonstrated the good accuracy and precision of the method, which is suitable for use in pharmacokinetic and bioavailability studies. The method was applied to samples from an experiment in a healthy volunteer treated with a single oral dose of 75 mg of brofaromine hydrochloride. Plasma profiles before and after enzymic hydrolysis showed that about one-third of the total brofaromine in plasma and practically all of the major metabolite (O-desmethylbrofaromine) were present in the conjugated form.

INTRODUCTION

Brofaromine [4-(7-bromo-5-methoxy-2-benzofuranyl)piperidine hydrochloride] is a new selective monoamine oxidase-A inhibitor [1]. It is extensively metabolized in humans, mainly by oxidative demethylation. Previously reported methods for its determination include gas chromatography (GC) after formation of a carbamate [2] and high-performance liquid chromatography after formation of a fluorescent derivative with NBD chloride (4-chloro-7-nitrobenzofurazan) [3]. Neither method included the O-desmethyl metabolite.

A sensitive GC assay for the simultaneous determination of the parent com-

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pound and its O-desmethyl metabolite in plasma and urine, before and after enzymic hydrolysis, was developed and validated.

EXPERIMENTAL

Reagents and chemicals

Brofaromine hydrochloride (I) ($C_{14}H_{16}BrNO_2 \cdot HCl$, mol. mass 346.65), its O-desmethyl metabolite (III) ($C_{13}H_{14}BrNO_2 \cdot HCl$, mol. mass 332.63) and an internal standard (V) ($C_{13}H_{14}BrNO \cdot HCl$, mol. mass 316.63) were all synthesized by Ciba-Geigy (Basle, Switzerland) and used as solutions in distilled water (for structures see Fig. 1). Sodium carbonate solution (0.1 *M*) and sodium hydroxide solution (1 *M*) were both laboratory grade. Diethyl ether, dichloromethane and *n*-hexane (all Fluka, Buchs, Switzerland) were distilled over a 1-m Vigreux column. Heptafluorobutyric anhydride (HFBA, Ventron, Karlsruhe, F.R.G.), acetate buffer pH 5.12 (1.795 mol sodium acetate and 0.825 mol acetic acid per litre) and *β*-glucuronidase–arylsulphatase solution were from Boehringer (Mannheim, F.R.G.).

Procedure

A 1-ml plasma or urine sample, 0.25 ml of internal standard solution (0.79 nmol), 2 ml of 0.1 *M* sodium carbonate and 4 ml of diethyl ether–dichloromethane

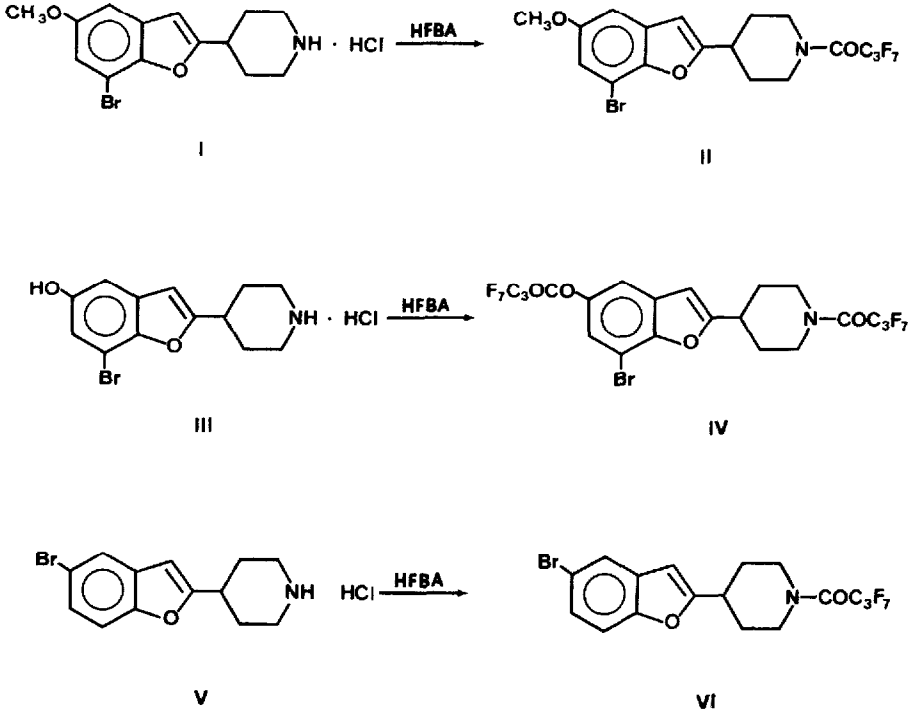

Fig. 1. Structures of brofaromine (I), its O-desmethyl metabolite (III), the internal standard (V) and the corresponding HFB derivatives (II, IV, VI).

(4:1, v/v) were shaken for 10 min at 150 rpm on a mechanical rotary shaker (Infors, Basle, Switzerland). After brief centrifugation the organic phase was transferred to a clean vial and evaporated to dryness under a stream of nitrogen at 40°C. To the dry residue, 0.3 ml of diethyl ether and 0.05 ml of HFBA were added. The vial was stoppered, briefly mixed and left to react at room temperature for 90 min. Excess reagent and solvent were removed by evaporation to dryness under a stream of nitrogen at 40°C. The residue was redissolved in 2 ml of *n*-hexane, and aliquots of 3–5 μl were injected into the gas chromatograph.

Hydrolysis

Hydrolysis of conjugates was optimized by incubation of urine as well as plasma samples, obtained from a volunteer who had been treated with an oral dose of brofaromine hydrochloride, for various time periods and with various amounts of enzyme.

Urine. A 0.1-ml sample of urine was diluted with 0.7 ml of distilled water, and 30 μl of β-glucuronidase–arylsulphatase solution and 250 μl of acetate buffer (pH 5.12) were added, mixed and incubated in a water bath (agitated) at 37°C for 15 h. After cooling, the samples were then adjusted with 1 *M* sodium hydroxide to pH 7.0 and processed as described above.

Plasma. To 1 ml of plasma were added 30 μl of β-glucuronidase–arylsylphatase solution and 250 μl of acetate buffer (pH 5.12). The solution was mixed and incubated as described above for urine samples.

Gas chromatographic conditions

The GC analysis was carried out on a Pye 304 instrument equipped with a ^{63}Ni electron-capture detector. The column was Pyrex glass (1.5 m × 4 mm I.D.) packed with 3% OV-17 on Supelcoport, 100–120 mesh. Temperatures were: column oven, 250°C; injector, 250°C; detector, 350°C. The nitrogen carrier gas flow-rate was 40 ml/min. A chromatogram of blank plasma extract, spiked with internal standard only, illustrates that no biological constituents interfere with the quantitation of brofaromine and its metabolite (Fig. 2). Chromatograms of spiked plasma and urine sample extracts are shown in Fig. 3. The retention times under the above conditions were 8.42 min for brofaromine, 3.65 min for the O-desmethyl metabolite and 4.74 min for the internal standard.

Chromatograms of plasma and urine samples from a human volunteer, who was treated with an oral dose of 75 mg of brofaromine hydrochloride, are presented in Fig. 4.

Stability

Plasma and urine samples remain unchanged for at least 2 months stored at −20°C in the dark. Stock solutions of brofaromine hydrochloride, its metabolite and the internal standard in water are stable at 4°C for at least 30 days. The derivatives, in hexane, are stable at 4°C for at least 10 days.

Extraction

The extractability from plasma was optimized by preparing spiked plasma samples as follows.

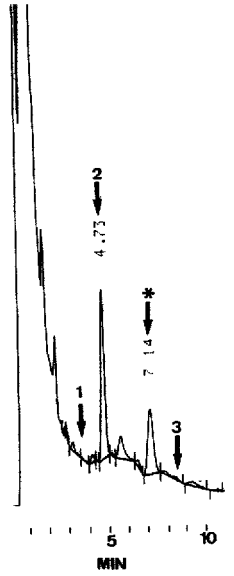

Fig. 2. Chromatogram of a blank plasma sample, spiked with 0.79 nmol of internal standard (2). The positions of the O-desmethyl metabolite (1) and brofaromine (3) are marked. The peak marked with an asterisk is an unknown constituent of plasma.

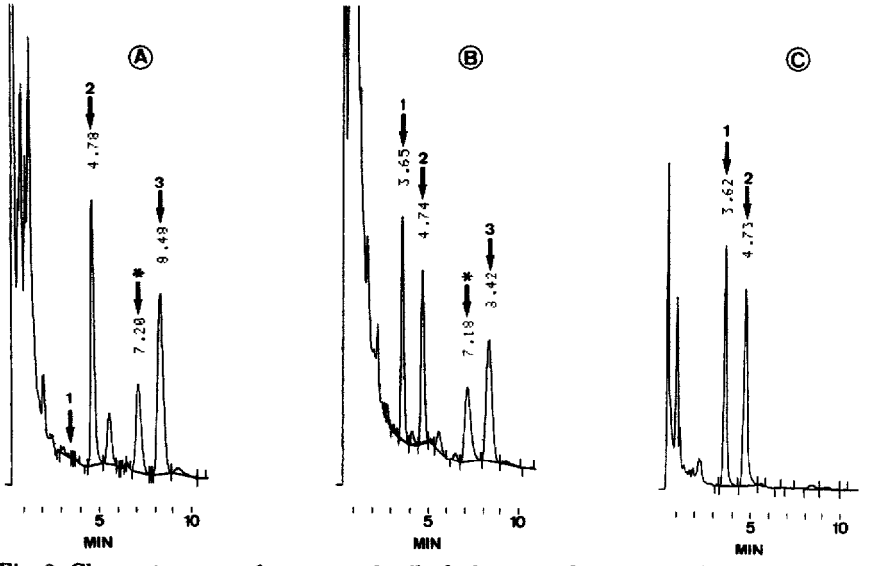

Fig. 3. Chromatograms of extracts of spiked plasma and urine samples: (A) 0.79 nmol of internal standard (2) and 0.87 nmol of brofaromine hydrochloride (3) in plasma; (B) 0.60 nmol of O-desmethyl metabolite (1), 0.79 nmol of internal standard (2) and 0.58 nmol of brofaromine hydrochloride (3) in plasma (measured after hydrolysis); (C) 1.20 nmol of O-desmethyl metabolite (1) and 0.79 nmol of internal standard (2) in urine (measured after hydrolysis). The peak marked with an asterisk is an unknown constituent of plasma.

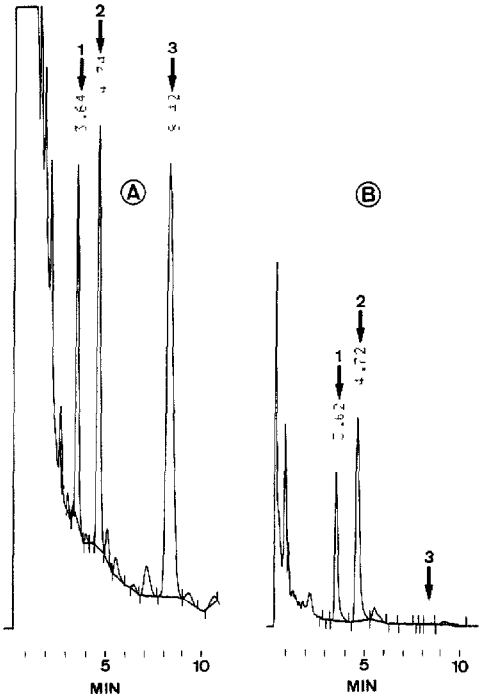

Fig. 4. Chromatograms of extracts of plasma and urine samples from a volunteer treated with an oral dose of 75 mg of brofaromine hydrochloride. (A) Plasma sample (2 h after administration), containing the equivalent of 0.79 nmol/ml internal standard (2), 1.46 nmol/ml brofaromine (3) and 0.46 nmol/ml O-desmethyl metabolite (1) (after enzymic hydrolysis). (B) Urine sample (collected 6–12 h after administration), containing the equivalent of 0.79 nmol per sample of internal standard (2), no measurable concentration of brofaromine (3) and the equivalent of 0.75 nmol/0.01 ml O-desmethyl metabolite (1) (after enzymic hydrolysis).

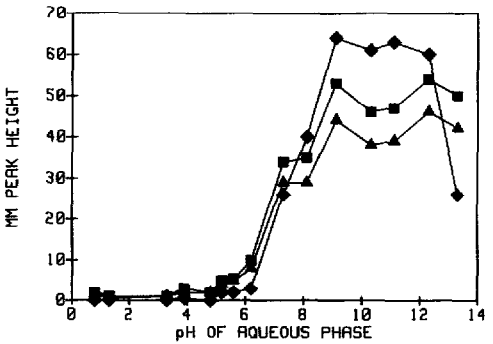

Fig. 5. pH dependency of the liquid–liquid extraction from plasma. ▲ = Brofaromine; ◆ = O-desmethyl metabolite; ■ = internal standard.

To 0.5 ml blank plasma, 7.2 nmol of brofaromine hydrochloride, 7.5 nmol of O-desmethyl metabolite and 7.9 nmol of the internal standard were added as aqueous solutions. The pH values of the samples were adjusted to values in the range 0.8–13.3, using 0.1 and 1 *M* solutions of sodium hydroxide or hydrochloric acid. The

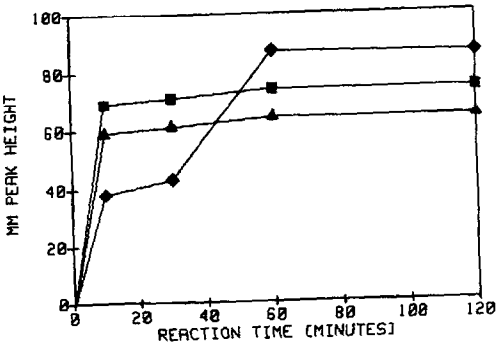

Fig. 6. Kinetics of the acylation reation with HFBA. ▲ = Brofaromine; ♦ = O-desmethyl metabolite; ■ = internal standard.

samples were then extracted and derivatized as described in *Procedure*, and chromatographed.

The pH-dependency is very similar for the extraction of brofaromine and the internal standard. Maximum extractability is achieved between pH 9 and 12. The O-desmethyl metabolite, however, is not extractable at highly basic pH (Fig. 5).

Derivatization

The reaction of the three compounds with HFBA was optimized. The maximum response for all three compounds was achieved after reaction for 60 min at room temperature (Fig. 6). With brofaromine and the internal standard the reaction is practically finished after 10 min, but the reaction with the metabolite needs at least 60 min for completion. This is probably due to the additional reactive group (phenolic OH). Thus, the standard reaction time was set at 90 min. The structures of the derivatives were verified by mass spectrometry.

RESULTS AND DISCUSSION

Calibration curves

A calibration curve for plasma determinations was prepared by spiking blank plasma samples with solutions of brofaromine hydrochloride and metabolite (0–1.45 nmol per sample). The samples were then processed as described. The peak heights of the brofaromine and metabolite derivatives were divided by the peak height of the internal standard derivative, and the ratio (H_x) was plotted against the initial concentrations. Calculation of the linear least-squares regression ($y=a+bx$) resulted in a coefficient of correlation of 0.9999 and an estimated standard deviation of 0.0101 for brofaromine. The calculation of the quadratic least-squares regression ($y=a+bx+cx^2$) resulted in a coefficient of correlation of 0.9999 and an estimated standard deviation of 0.0099 for the O-desmethyl metabolite.

Recoveries, precision and limit of quantitation

The recovery and precision of the described assay for brofaromine and its metabolite were evaluated by analysing spiked plasma and urine samples. Eighteen

TABLE I

PARAMETERS OF THE LINEAR REGRESSION ($y=a+bx$) BETWEEN GIVEN AND FOUND CONCENTRATIONS ($n=18$)

Parameter	Brofaromine	O-Desmethyl metabolite
Coefficient of correlation	0.9995	0.9991
Estimated standard deviation	0.0078	0.0121
Intercept (a)	0.0071	−0.0018
Slope (b)	0.9885	1.1015

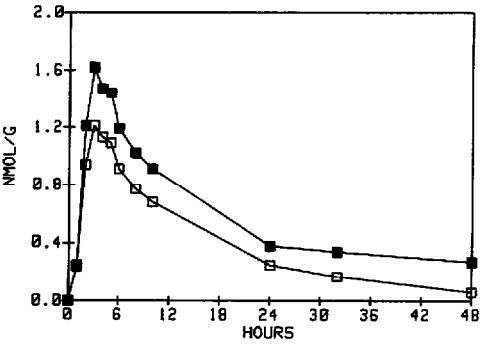

Fig. 7. Plasma concentrations of unchanged brofaromine measured before (□) and after (■) enzymic hydrolysis in a healthy volunteer treated with a single oral dose of 75 mg of brofaromine hydrochloride in form of a film-coated tablet.

samples with brofaromine and metabolite concentrations between 0.035 and 0.722 nmol per sample were prepared. Each sample was analysed in triplicate. The differences between given and found concentrations ranged from 0 to 14% in the concentration range 0.11–0.72 nmol per sample, and 26% at the 0.035 nmol level in plasma. The coefficient of variation ranged from 0 to 2.2% for brofaromine and from 1.3 to 6.7% for the metabolite, over the entire concentration range. The parameters of the linear regression ($y=a+bx$) between given and found concentrations are given in Table I. The limit of quantitation is ca. 0.035 nmol per sample for both compounds.

Application

One healthy volunteer was treated with a single oral dose of 75 mg of brofaromine hydrochloride in form of a film-coated tablet. The plasma samples were measured before and after enzymic hydrolysis.

The concentration of unchanged brofaromine reached a maximum of 1.2 nmol/g before hydrolysis and 1.62 nmol/g after hydrolysis (Fig. 7). The concentration of the desmethyl metabolite reached only 0.06 nmol/g before hydrolysis but 0.89 nmol/g after hydrolysis (Fig. 8). Thus, about one-third of the total brofaromine

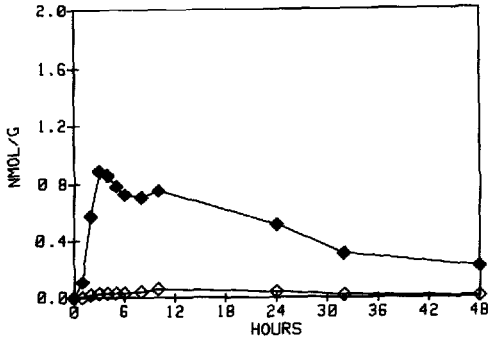

Fig. 8. Plasma concentrations of the O-desmethyl metabolite measured before (◇) and after (◆) enzymic hydrolysis in a healthy volunteer treated with a single oral dose of 75 mg of brofaromine hydrochloride in form of a film-coated tablet.

in plasma and practically all of the desmethyl metabolite were present in the conjugated form.

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