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Note

Gas chromatographic determination of stobadine, N-desmethylstobadine and stobadine N-oxide in rat liver microsomal incubations

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Stobadine, (–) -*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-*b*]indole (I), is a prospective antiarrhythmic and cardioprotective agent [1–3] derived from the γ-carboline neuroleptic and antidepressant carbidine [4,5] as its optically active stereoisomer. Stobadine was in rat liver microsomal preparations mainly metabolized to N-desmethylstobadine (II) and stobadine N-oxide (III) [6]. Both metabolites were found in rat urine after intraperitoneal (i.p.) administration of the drug [7]. The structures of stobadine and its two metabolites are shown in Fig. 1.

Previously reported methods for the quantification of stobadine in biological fluids and tissues include spectrofluorimetry [8] and radiometric determinations based on selective extraction of the drug [9]. A selected-ion monitoring assay by gas chromatography–mass spectrometry (GC–MS) was described for the determination of structurally related endogenous tetrahydro-β-carbolines in tissue homogenates [10].

As we intended to investigate in vitro N-dealkylation and N-oxidation of stobadine, we required a method of analysis whereby both of these pathways could be studied simultaneously. In this paper we describe a gas chromatographic assay for stobadine, N-desmethylstobadine and stobadine N-oxide. The sensitivity of the method was sufficient for measuring hepatic stobadine N-demethylase and stobadine N-oxidase activities in vitro.

EXPERIMENTAL

Materials

Stobadine (I) dihydrochloride [1–3], racemic N-desmethylstobadine, (±)-*cis*-8-methyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-*b*]indole (II) [11] and the

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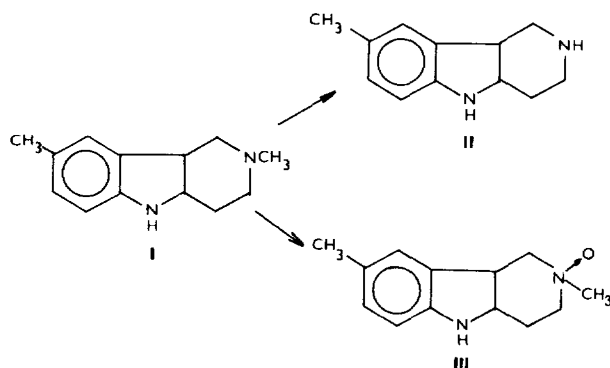

Fig. 1. Metabolic conversion of stobadine (I) to N-desmethylstobadine (II) and stobadine N-oxide (III).

racemic N-ethyl analogue of stobadine, (±) -*cis*-2-ethyl-8-methyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-*b*]indole (IV) [11], used as an internal standard, were synthesized at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, (Prague, Czechoslovakia). Stobadine N-oxide, (−)-cis-2,8-dimethyl-2-oxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-*b*]indole (III), was synthesized as reported elsewhere [6]. Trifluoroacetic anhydride (TFA) was purchased from Fluka (Buchs, Switzerland) and titanium(III) chloride (ca. 15% solution in 3 *M* hydrochloric acid) from E. Merck (Darmstadt, F.R.G.). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

Gas chromatography

GC was performed on a CHROM 5 gas chromatograph (Laboratory Instruments, Prague, Czechoslovakia) equipped with a flame ionization detector. The glass column (2.5 m × 3 mm I.D.) was packed with 3% OV-17 on Chromosorb W (80–100 mesh). The column oven was operated isothermally at 200 °C or 220 °C. The temperature of the injection port was 320 °C and that of the detector 340 °C. The carrier gas was nitrogen at a flow-rate of 30 ml/min.

Gas chromatography–mass spectrometry

A Jeol JMS-D 100 combined gas chromatograph–mass spectrometer was employed. GC separations were accomplished under the above-mentioned conditions using helium as the carrier gas. The MS conditions were: ion source temperature, 260 °C; emission current, 300 μA; electron beam energy, 23 eV; separator temperature, 270 °C.

Derivatization

TFA (100 μl) was added to 2.5–50 nmol of I, II and IV in separate 10-ml glass tubes. The tubes were being tightly stoppered and heated for 5, 15, 30 and 60 min in a 50 °C water-bath. After cooling, the excess of the reagent was evaporated under nitrogen, the remainder being dissolved in methanol (50 μl) and subjected

to temperature-programmed GC–MS analysis [6]. The trifluoroacetylation of the compounds studied was complete after 15 min.

Microsomal incubations

Male Wistar rats (220–270 g) pre-treated with sodium phenobarbital for four days (3×80 mg/kg+1×40 mg/kg, i.p.) were used as liver donors. Liver microsomes were prepared according to the method of Cho et al. [12]. A microsomal suspension equivalent to 0.5 g of liver (wet weight) was incubated aerobically at 37°C in a total volume of 5 ml of 0.15 *M* potassium phosphate buffer (pH 7.5) containing 1.15% of potassium chloride, 1.5 μmol of NADP, 17.5 μmol of glucose 6-phosphate, 20 μmol of magnesium chloride, 5 I.U. of glucose 6-phosphate dehydrogenase and 5 μmol of stobadine dihydrochloride. Incubations were started by the addition of the liver preparation.

Determination of stobadine and N-desmethylstobadine

The metabolic reaction was terminated by transferring the entire contents of the flasks into extraction tubes containing 0.5 ml of 5 *M* hydrochloric acid; sodium chloride (0.5 g) was added and the precipitated protein was removed by centrifugation. The pellets were washed with 1.15% potassium chloride solution (1 ml) and, after the addition of 9 nmol of the internal standard (IV) and mixing, the combined aqueous solutions and washings were extracted with dichloromethane (6 ml). The organic layer was discarded and, after adjustment of the pH to 10 with 5 *M* sodium hydroxide solution, the remaining aqueous phase was extracted with dichloromethane (3×3 ml). The combined dichloromethane extracts were filtered through silanized glass-wool and evaporated to dryness under a stream of nitrogen. The residues were derivatized as described above using a 60-min reaction time, and subjected to GC and GC–MS analysis.

To determine N-desmethylstobadine (II) in the presence of a large excess of the parent drug in the microsomal incubations, an *n*-hexane washing step (6 ml) at pH 10 was included in the above procedure prior to the addition of the internal standard.

Determination of stobadine N-oxide

The aqueous phases remaining after the extraction of unchanged stobadine and its N-desmethyl metabolite were mixed with hydrochloric acid 5 *M* (1 ml) and 15% titanium(III) chloride solution (1 ml). After keeping at ambient temperature overnight, the mixture was adjusted to pH 10 with 5 *M* sodium hydroxide solution and extracted with dichloromethane (2×3 ml) in the presence of the internal standard (IV, 9 nmol). The combined organic extracts were filtered through silanized glass-wool and evaporated to dryness under a stream of nitrogen. The residues were dissolved in methanol (50 μl) and analysed underivatized.

Calibration graphs

Calibration graphs were prepared by adding various amounts of I, II or III and a constant amount (9 nmol) of the internal standard (IV) to incubation mix-

tures without cofactors. These standard samples were quenched immediately and processed through the assay.

Recovery studies

Control incubation mixtures containing I (5 nmol/ml) or II (10 nmol/ml) were carried through the above extraction procedure without the addition of internal standard. Compound IV (9 nmol) dissolved in dichloromethane was then added to the biological extracts and the organic solutions were evaporated to dryness under a stream of nitrogen. The subsequent analysis was carried out as described above. Recoveries of I and II were calculated by comparing the peak-height ratios with those obtained when compounds I, II and IV, dissolved in dichloromethane, were analysed without the extraction procedure. Similarly, the recovery of IV at 5 nmol/ml was determined by employing the above procedure with I as the internal standard.

RESULTS AND DISCUSSION

Prior to the extraction of I and II the biological samples were washed with dichloromethane at acidic pH. This procedure reduced the interferences of endogenous lipophilic constituents without a loss of the drug and metabolites. After alkalinization to pH 10 the samples were extracted with further portions of dichloromethane. At the pH used, stobadine ($pK_{a2} = 8.71 \pm 0.09$) [13] and its N-ethyl congener IV were readily extractable, the absolute recoveries at 5 nmol/ml being $99.3 \pm 1.6\%$ and $99.6 \pm 2.0\%$ [mean ± standard error of the mean (S.E.M.), $n = 6$], respectively. The overall recovery of II during sample preparation at 10 nmol/ml was $81.3 \pm 2.9\%$ (mean ± S.E.M., $n = 6$).

The compounds extracted were treated with TFA and subjected to GC and GC–MS. The introduction of one TFA group under the conditions employed was confirmed from the mass spectra of the derivatives of I and IV, which showed corresponding molecular ions at m/z 298 and 312, respectively. The molecular ion at m/z 380 in the mass spectrum of II corresponds to the introduction of two TFA groups.

Fig. 2 depicts a typical chromatogram of the rat liver microsomal incubation showing a separation of I, IV and II. No interfering peaks due to endogenous substances in the incubation mixture were observed.

The overall hydrophilic character of III made its extraction into the organic phase impossible during the procedure employed. A method frequently used for the quantification of polar N-oxides in biological samples is based on titanium (III) chloride reduction followed by analysis of the extractable amine formed [14–17]. This method was used for the determination of stobadine N-oxide in the aqueous phase remaining after the extraction of the parent drug. The background in the N-oxide assay due to unextracted stobadine was negligible. The gas chromatogram of a second extract after reduction of an incubation mixture with titanium(III) chloride is shown in Fig. 3.

Calibration graphs prepared for determining I, II and III in rat liver microsomal incubations were linear (regression coefficient > 0.998) over the concentration

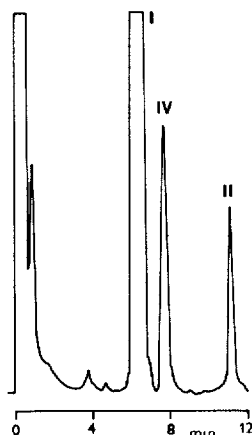

Fig. 2. Chromatogram of an extract from a rat liver microsomal preparation spiked with I (250 nmol) and II (15 nmol) in the presence of the internal standard IV (9 nmol) after TFA derivatization. Column temperature: 200°C.

range 0.5–10.0 nmol/ml. Other experiments showed that this range of linearity could be extended to at least 100 nmol/ml.

To evaluate the overall reproducibility of the assay, several replicate microsomal samples containing known concentrations of I, II and III were analysed (Table I). For all compounds at the level of 1.0 nmol/ml the coefficient of variation was less than 10%. The metabolic studies performed so far have involved higher concentrations, however (see Table II).

In in vitro experiments very small amounts of the metabolically produced II were to be determined in the presence of excess amounts of the parent drug. To avoid the injection of large amounts of I into the GC column, an *n*-hexane washing step was included in the extraction procedure used (see Experimental). As expected in view of the high partition coefficient of [^{3}H]stobadine [9], above

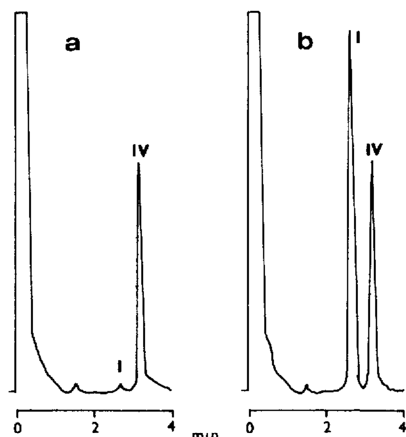

Fig. 3. Chromatogram of a second extract after titanium (III) chloride reduction of a rat liver microsomal preparation containing 1 m*M* I in the presence of the internal standard IV (9 nmol). (a) Without III; (b) with 22.5 nmol of III. Column temperature: 220°C.

TABLE I

REPEATABILITY OF THE DETERMINATION OF STOBADINE (I), N-DESMETHYLSTOBADINE (II) AND STOBADINE N-OXIDE (III) IN MICROSOMAL INCUBATIONS

Each compound at two concentrations was analysed in five independent samples ($n=5$).

Compound	Theoretical concentration (nmol/ml)	Observed concentration (mean ± S.E.M.) (nmol/ml)	Coefficient of variation (%)
I	1.0	1.11 ± 0.08	7.2
II		0.94 ± 0.09	9.6
III		0.92 ± 0.08	8.7
I	10.0	10.14 ± 0.39	3.8
II		10.21 ± 0.52	5.1
III		9.87 ± 0.42	4.3

TABLE II

METABOLIC N-DEMETHYLATION AND N-OXIDATION OF STOBADINE BY PHENOBARBITAL-INDUCED RAT LIVER MICROSOMES

Substrate concentration: 1.0 m*M*. Results are mean values ± S.E.M. for three rats.

Metabolic reaction	Activity (nmol per 15 min per gram wet liver)
N-Demethylation	64.1 ± 14.0
N-Oxidation	549.3 ± 62.2

95% of I at 1000 nmol/ml was removed by single *n*-hexane extraction at pH 10, while the absolute recovery of the more polar II at 10 nmol/ml in successive triple dichloromethane extraction remained above 50%, which is in accord with the low distribution ratio of II between the aqueous phase and *n*-hexane ($D=0.25$, pH 10, $V_{org}/V_{aq}=1$). A high selectivity with respect to the metabolic products was achieved in the *n*-hexane extraction of [^{3}H]stobadine from alkalinized (2 *M* sodium carbonate solution) biological samples in experiments in vivo [9]. To correct any losses of II before the addition of the internal standard, calibration graphs prepared simultaneously from standard samples processed in the same manner as the unknowns were used. This procedure allowed II to be determined at 1.0 and 10.0 nmol/ml in the presence of 1 m*M* I in rat liver microsomal incubations with coefficients of variation of 13.3 and 9.1% ($n=5$), respectively. Investigation of the mass spectra of trifluoroacetylated I, II and IV [6] has suggested that the selectivity and sensitivity of the assay can, if needed, be further improved by GC–MS analysis with selected-ion monitoring.

The assay was applied to the analysis of microsomal incubations obtained from rats pre-treated with phenobarbital. The results (Table II) indicate that the method described can be used successfully to measure hepatic stobadine N-demethylase and stobadine N-oxidase activities in vitro.

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