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SELECTIVE AND SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE METABOLITES OF NOMIFENSINE IN HUMAN PLASMA

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SUMMARY

A selective high-performance liquid chromatographic method for the determination of the three metabolites of nomifensine in human plasma is described. All metabolites and the internal standard, mexiletine, are extracted with diethyl ether and then back-extracted into an acidic aqueous phase. After subsequent extraction into diethyl ether the metabolites are analysed by high-performance liquid chromatography. A reversed-phase C_{18} column is used with a mobile phase of dioxane—methanol—potassium phosphate buffer (pH 2.25). The sensitivity of the method is 0.007 μmol/l for all metabolites. Extraction efficiencies are 84.6%, 75.8%, and 78.2% for 4′-hydroxynomifensine, 4′-hydroxy-3′-methoxynomifensine and 3′-hydroxy-4′-methoxynomifensine, respectively. The reproducibility of the method is good, the coefficients of variation (%) varying between 2.1% and 9.9% in the concentration range 0.05—1.00 μmol/l. The procedure was applied to human plasma samples from a volunteer who had received a single oral dose of nomifensine. The method is accurate and sensitive for pharmacokinetic studies on the metabolites of nomifensine.

INTRODUCTION

Nomifensine is a psychotropic agent with demonstrated antidepressant properties [1]. The chemical structure of nomifensine is 8-amino-1,2,3,4-tetrahydro-2-methyl-4-phenylisoquinoline (Fig. 1). The first step in the metabolism of nomifensine is hydroxylation; the phenyl ring is hydroxylated in the 3′ and 4′ positions. So the first and probably the most important metabolite is 4′-hydroxynomifensine (M_1) [2]. In the next phase the hydroxyl groups are methylated, resulting in the formation of 4′-hydroxy-3′-methoxynomifensine (M_2) and 3′-hydroxy-4′-methoxynomifensine (M_3) [2]. Besides these three main metabolites (M_1, M_2 and M_3) four further metabolites are formed, but in

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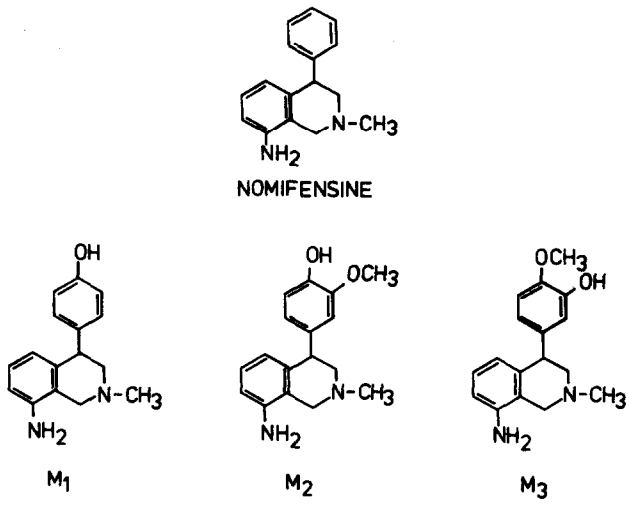

Fig. 1. Structures of nomifensine and its three main metabolites M_1, M_2 and M_3.

negligible amounts [3]. All metabolites are conjugated in the last phase of the metabolism [4].

An accurate and selective methodology for the measurement of the parent drug and its metabolites in human body fluids is a prerequisite for detailed pharmacokinetic studies. A number of methods for the determination of nomifensine have been published [5–9]. Uihlein and Hajdú [10] have described a liquid chromatographic method for the analysis of nomifensine, metabolite M_1 and the sum of metabolites M_2 and M_3. The sensitivity and selectivity of this procedure are insufficient for pharmacokinetic studies. A quantitative thin-layer chromatographic method for the measurement of nomifensine and its three metabolites in human urine has also been published [11]. However, no method for the quantitation of all three main metabolites of nomifensine, M_1, M_2 and M_3, in human plasma has been described.

This paper describes a selective, sensitive and accurate high-performance liquid chromatographic (HPLC) procedure for the detection of all three principal metabolites of nomifensine, M_1, M_2 and M_3, in non-conjugated form in human plasma. The procedure was used to monitor plasma concentrations of these metabolites in a volunteer who had received a single oral dose of nomifensine.

EXPERIMENTAL

Reagents and chemicals

The following reagents were used: 4-hydroxynomifensine hydrogen maleate (M_1), 4-hydroxy-3-methoxynomifensine (M_2) and 3-hydroxy-4-methoxynomifensine (M_3) were gifts from Hoechst (Frankfurt am Main, F.R.G.). Mexiletine hydrochloride was obtained from Boehringer (Mannheim, F.R.G.). Diethyl ether, dioxane and methanol were of analytical-reagent grade (Merck, Darmstadt, F.R.G.).

A solution of mexiletine in distilled water (20 μmol/l) was used as the internal standard.

Working metabolite solutions contained 10 or 100 μmol/l in 50% methanol. Drug plasma standards were prepared by spiking blank control plasma with appropriate microlitre volumes of each working metabolite solution to obtain seven plasma standards with the following concentrations of each metabolite: 0.025, 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 μmol/l.

Extraction procedure

To a 1.0-ml plasma sample, 125 μl of mexiletine solution (20 μmol/l) were added. The plasma was made alkaline by adding 1 ml of 0.1 *M* sodium tetraborate buffer (pH 9.0). The metabolites M_1, M_2 and M_3 and the internal standard were extracted with 5 ml of diethyl ether by shaking for 20 min. After centrifugation (1200 *g*) the diethyl ether layer was transferred to a new clean tube containing 1 ml of 0.2 *M* hydrochloric acid. The mixture was shaken for 20 min. The diethyl ether phase was separated by centrifugation (1200 *g*) and aspirated. The acidic layer was made alkaline with 3 ml of 0.1 *M* sodium tetraborate buffer (pH 9.0). The metabolites M_1, M_2 and M_3 and the internal standard were extracted with 5 ml of diethyl ether by shaking for 20 min. The organic phase was evaporated at 40°C under a gentle stream of pure nitrogen. The residue was reconstituted in 100 μl of phosphoric acid (0.05 *M*), and 50 μl were injected into the chromatograph. The concentrations of all three metabolites in the plasma samples were determined from a calibration curve of peak height ratio (drug/internal standard) versus drug concentration in plasma standards carried through this procedure.

Chromatographic system

The determinations were carried out using the following chromatographic system: SP 8700 solvent-delivery system with SP 8750 organizer (Spectra-Physics, Santa Clara, CA, U.S.A.); Rheodyne injector with 50-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.); variable-wavelength UV detector SF 773 set at 210 nm (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). The reversed-phase column was a 10-μm μBondapak C_{18}, 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.). The solvent used was methanol—dioxane—0.01 *M* potassium phosphate buffer (pH 2.25) (6.5:7:86.5) and the flow-rate was 2.2 ml/min. Chromatograms were recorded with a laboratory potentiometric recorder.

Application of the method

The formation of the three metabolites of nomifensine, M_1, M_2 and M_3, was studied in a healthy volunteer who had taken 100 mg of nomifensine in capsule form orally. The volunteer fasted overnight and received a breakfast 3 h after taking the drug. Seventeen blood samples were taken, up to 24 h after administration of the drug. Plasma was promptly separated and frozen at −60°C until analysis.

RESULTS AND DISCUSSION

Chromatograms of extracts from blank plasma, the plasma sample of a volunteer after a single dose of nomifensine and blank plasma spiked with

0.50 μmol/l of metabolites M_1, M_2 and M_3 are illustrated in Fig. 2A, B and C, respectively. The metabolites M_1, M_2 and M_3 and the internal standard, mexiletine, were well separated with retention times of 4.00, 4.50, 5.60 and 6.60 min, respectively. The HPLC method showed a linear increase in response over the concentration range 0.01—1.00 μmol/l in plasma for all three metabolites (Fig. 3). A plot of peak height ratio against metabolite concentration gave a linear calibration curve for each metabolite as well. The equations of the

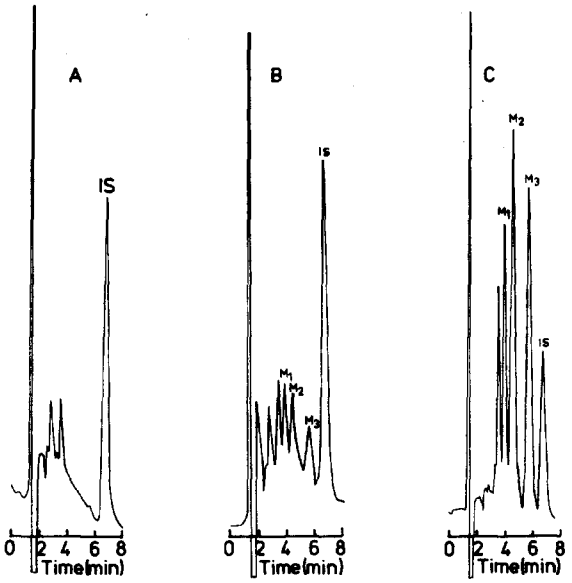

Fig. 2. Liquid chromatograms of the extracts from blank plasma (A), a plasma sample of a volunteer 50 min after a single 100-mg dose of nomifensine (B) and blank plasma spiked with 0.50 μM metabolites M_1, M_2 and M_3 (C). The concentrations of the metabolites M_1, M_2 and M_3 are 0.070, 0.050 and 0.045 μmol/l, respectively. IS = internal standard, mexiletine. For chromatographic conditions, see text.

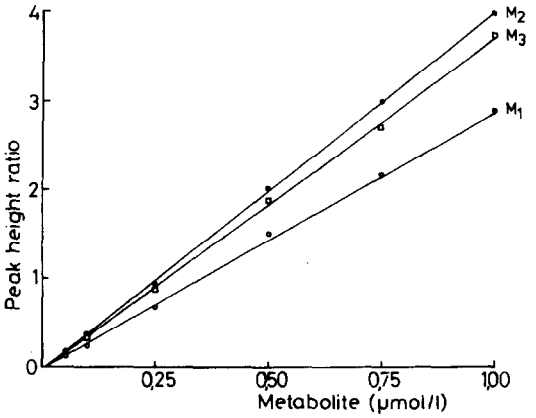

Fig. 3. Calibration graphs for metabolites M_1, M_2 and M_3. Peak height ratios of metabolites to those of the internal standard are plotted against metabolite concentration in plasma. The correlation coefficients of the lines are $r > 0.999$, 0.999 and 0.998 for M_1, M_2, and M_3, respectively.

calibration curves were $y = 2.93x - 0.04$, $y = 4.12x - 0.06$ and $y = 3.79x - 0.06$ (y = peak height ratio, metabolite/internal standard and x = metabolite concentration) for M_1, M_2, M_3, respectively. When defined as a signal level exceeding three times the background noise, the detection limit of the present HPLC method is 0.007 μmol/l for all three metabolites.

The precision was assessed by multiple analyses of six standard plasma pools in the concentration range 0.05—1.00 μmol/l for all three metabolites. The coefficients of variation for intra-assay variability of metabolites are given in Table I: they varied from 2.1% to 9.9% in the concentration range studied. The results demonstrate the high accuracy and reproducibility of the method.

From a comparison of metabolite peak heights obtained from direct injection of aqueous solutions and from samples carried through the assay procedure, the extraction efficiencies were estimated as 84.6 ± 1.0%, 75.8 ± 1.4% and 78.2 ± 1.4% (± S.E., $n = 8$) for M_1, M_2 and M_3, respectively (Table II). The recoveries were calculated over the concentration range 0.05—1.00 μmol/l.

To extract nomifensine metabolites M_1, M_2 and M_3, four different extraction solvents were tried: ethyl acetate, diethyl ether, hexane and dichloromethane. The highest recovery was found with diethyl ether for all three metabolites. The optimum pH value for the extraction of all three metabolites was found to be 9. This is supported by the maximum partition coefficients in octanol—water

TABLE I

INTRA-ASSAY REPRODUCIBILITY ($n = 8$) OF HPLC FOR THE DETERMINATION OF NOMIFENSINE METABOLITES IN HUMAN PLASMA

Concentration (μmol/l)	Coefficient of variation (%)		
	M_1	M_2	M_3
0.05	4.5	7.5	9.9
0.10	7.3	6.8	8.3
0.25	5.2	6.1	8.0
0.50	5.5	4.7	6.9
0.75	2.2	2.4	2.1
1.00	6.4	3.8	4.7

TABLE II

DETERMINATION OF NOMIFENSINE METABOLITES IN HUMAN PLASMA ($n = 8$)

Concentration (μmol/l)	Extraction efficiency (mean ± S.E. %)		
	M_1	M_2	M_3
0.05	85.5 ± 1.4	69.7 ± 4.1	79.7 ± 3.4
0.10	83.1 ± 3.1	61.3 ± 2.0	65.5 ± 3.5
0.25	86.5 ± 0.8	81.2 ± 1.1	81.2 ± 2.2
0.50	78.3 ± 1.7	81.3 ± 1.2	87.1 ± 1.9
0.75	82.7 ± 1.2	75.7 ± 0.8	74.0 ± 0.7
1.00	91.6 ± 2.7	85.6 ± 1.4	81.5 ± 2.0
Mean	84.6 ± 1.0	75.8 ± 1.4	78.2 ± 1.4

of 79 ± 9 for nomifensine, 50 for M_1 and 26 for M_2 at pH 8—10, as estimated by Sistovaris [11]. Back-extraction of the metabolites into an acidic aqueous phase almost purified the extract from the endogenous compound eluting just before M_1 (Fig. 2). Mexiletine was chosen as the internal standard because it was extracted well in the procedure used here and eluted later than all three metabolites, with a retention time of 6.60 min.

Several mobile phases were investigated before the final selection of the chromatographic conditions was made. Acetonitrile, tetrahydrofuran, methanol and dioxane were evaluated as organic components of the eluent. Acetonitrile in the mobile phase diminished the UV absorption of all metabolites, probably by a chemical reaction with the metabolites, resulting in poor detection. Acetonitrile, tetrahydrofuran and methanol did not sufficiently separate the metabolites M_1 and M_2. Dioxane was the only organic component that separated all three metabolites from each other. Replacing a part of dioxane by methanol in the mobile phase diminished the background noise markedly.

The interference of several psychotropic drugs with the present HPLC method was checked by injecting concentrated solutions of these compounds into the chromatographic system. From the tested substances chlordiazepoxide, perphenazine, desmethyldoxepin, thioridazine, sulpiride, mianserin, doxepin and oxazepam did not interfere. Caffeine was also tested and it eluted just before the metabolite M_1, so it might be the endogenous compound seen in the chromatograms of extracted human plasma (Fig. 2).

The parent drug, nomifensine, eluted after the metabolites and the internal standard with a retention time of 9.80 min. Nomifensine did not, therefore, interfere with the analysis of metabolites, but lengthened the total time for each chromatographic run. In principle it is possible to determine the parent drug, nomifensine, simultaneously with the metabolites, but a more accurate and sensitive method published earlier [9] is recommended for the analysis of nomifensine. Plasma concentrations of free metabolites M_1, M_2 and M_3 in a male volunteer who had received a single oral dose of 100 mg of nomifensine are shown in Fig. 4. The results clearly demonstrate that the sensitivity of the method is sufficient for pharmacokinetic studies.

In conclusion, the HPLC method described here is demonstrated to be selective and sensitive, and therefore suitable for pharmacokinetic studies on the metabolites M_1, M_2 and M_3. In preliminary experiments the method proved to be applicable to an urine analysis of nomifensine metabolites M_1, M_2 and

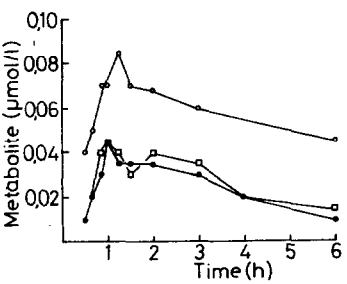

Fig. 4. Plasma profile of three non-conjugated metabolites M_1 (○), M_2 (●) and M_3 (□) in a volunteer after a single oral dose of 100 mg of nomifensine.

M_3, too. The method is currently in use for the determination of nomifensine metabolites in human plasma and urine after intravenous administration of the drug.

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