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Simultaneous determination of mexiletine and four hydroxylated metabolites in human serum by high-performance liquid chromatography and its application to pharmacokinetic studies

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ABSTRACT

A high-performance liquid chromatographic method has been developed for the simultaneous determination of mexiletine and its four hydroxylated metabolites in human serum. The method involves a single-step extraction of mexiletine, hydroxymethylmexiletine, *p*-hydroxymexiletine and their corresponding alcohols with diisopropyl ether–dichloromethane–propan-2-ol (2.5:1.5:0.5, v/v). Separation of the compounds on a deactivated Supelcosil LC_8-DB column is accomplished by high-performance liquid chromatography with ultraviolet detection at 203 nm. Overall the recovery of each compound is reproducible and greater than 75%. The lower limit of detection is 2 ng/ml for mexiletine and its metabolites. The application of the method is shown by measuring the concentrations in serum of mexiletine and its metabolites over 24 h in a healthy volunteer after a single intravenous injection of the drug and by monitoring serum concentrations in patients receiving long-term treatment by mouth of the drug.

INTRODUCTION

Mexiletine (MEX), 1-(2,6-dimethylphenoxy)-2-aminopropane, is a well tolerated and effective antiarrhythmic drug when given orally and has shown effectiveness against a variety of cardiac arrhythmias [1,2]. MEX is almost completely absorbed from the gastrointestinal tract and is extensively metabolized with < 10% of the dose excreted as the unchanged drug in urine. The major pathway of MEX biotransformation in humans is hydroxylation followed by secondary methylation, deamination, reduction and conjugation [3,4]. Hydroxymethylmexiletine (OHMeMEX) and *p*-hydroxymexiletine (*p*-OHMEX) are considered as major metabolites of MEX [5,6]. Both metabolites and their corresponding alcohols (OHMeMEX-OL, *p*-OH MEX-OL, Fig. 1) are excreted in urine in the conjugated and free forms, accounting for up to 20%

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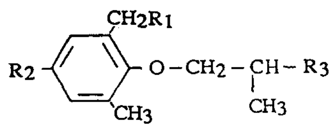

R1	R2	R3	
H	H	NH_2	MEX
OH	H	NH_2	OHMeMEX
H	OH	NH_2	p-OHMEX
OH	H	OH	OHMeMEX-OL
H	OH	OH	p-OHMEX-OL

Fig. 1. Structures of mexiletine (MEX) and its metabolites 1-(2-hydroxymethyl-6-methylphenoxy)-2-aminopropane (OHMeMEX), 1-(2,6-dimethyl-4-hydroxyphenoxy)-2-aminopropane (*p*-OHMEX), 1-(2-hydroxymethyl-6-methylphenoxy)-propan-2-ol (OHMeMEX-OL), 1-(4-hydroxy-2,6-dimethylphenoxy)-propan-2-ol (*p*-OHMEX-OL).

of the dose [7]. In serum samples from patients treated with the drug, OHMeMEX and *p*-OHMEX reach 40 and 9% of the MEX concentration (unpublished results).

Gas chromatography [8–12] and high-performance liquid chromatography (HPLC) [13–20] have been widely used for the determination of MEX in biological fluids. Few of these methods are easily adaptable for metabolite measurements. Farid and White [21] reported a sensitive gradient HPLC method with fluorescence detection for the simultaneous determination of MEX, OHMeMEX and *p*-OHMEX in human plasma. Another HPLC assay which separates the two hydroxy metabolites from parent drug in serum using UV detection has also been described [22]. The flow programming technique was applied to quantitative studies of OHMeMEX and *p*-OHMEX in human liver microsomes by Broly *et al.* [23]. More recently, Krämer *et al.* [24] developed an HPLC method under isocratic conditions for the simultaneous determination of MEX and the two main metabolites in serum. Quantitation of OHMeMEX, *p*-OHMEX and their corresponding alcohols in human serum has been achieved by a tedious method combining UV and amperometric detection [25]. No analytical method is available to determine MEX, OHMeMEX, *p*-OHMEX, OHMeMEX-OL and *p*-OHMEX-OL simultaneously.

This paper presents a sensitive, selective and reproducible HPLC assay for the simultaneous determination of MEX and its four hydroxylated metabolites in human serum for application to pharmacokinetic studies of MEX.

EXPERIMENTAL

Chemical and reagents

MEX hydrochloride, OHMeMEX oxalate, *p*-OHMEX hydrochloride, OHMeMEX-OL, *p*-OHMEX-OL oxalate and 1-(2,4-dimethylphenoxy)-2-aminopropane (KOE 768) as the internal standard (I.S.) were kindly supplied by Boehringer Ingelheim (Ingelheim, Germany). Dichloromethane, acetonitrile, methanol and 2-propanol were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

Instrumentation and conditions

The chromatographic system consisted of a solvent delivery pump (Model 2150, LKB, Bromma, Sweden), an injector (Model 7125, Rheodyne, Cotati, CA, USA), a variable-wavelength detector (Spectra 100, Spectra-Physics, San Jose CA, USA) and an integrator (Chromjet, Spectra-Physics). Separation was performed on a deactivated Supelcosil LC_8-DB column (25 cm × 4.6 mm I.D., 5 μm particle size) protected with a Supelguard LC_8-DB precolumn (2 cm × 4.6 mm I.D., 5 μm particle size) at room temperature. The mobile phase, prepared fresh daily, consisted of acetonitrile–methanol–0.5 *M* potassium dihydrogenphosphate (KH_2PO_4)–water (120:40:6:334, v/v). The pH was adjusted to 3.5 with 100 μl of orthophosphoric acid. The flow-rate was set at 1.2 ml/min. The column eluent was monitored at 203 nm using a detector range of 0.001 a.u.f.s. and a chart speed of 0.25 cm/min. The injection volume was 50 μl.

Standard solutions

Stock standard solutions of MEX, its metabolites and the I.S. were prepared by dissolving 100 mg of each compound in 100 ml of methanol. The solutions were stable for at least six months if stored at 4°C. Working standard solutions were prepared from the stock solutions by suitable dilutions with methanol to obtain final concentrations of 2 μg/ml for all metabolites and 6 μg/ml for MEX and the I.S.

Extraction procedure

A 0.5-ml aliquot of serum was introduced into a screw-capped glass centrifuge tube to which 50 μl of the working I.S. solution and 200 μl of borate buffer (pH 10) were added. The serum was then extracted with 4 ml of diisopropyl ether–dichloromethane–propan-2-ol (2.5:1.5:0.5, v/v) for 5 min. After centrifugation at room temperature for 5 min at 3000 *g*, the sample was frozen at -14°C in a methanol bath. The organic phase was transferred into a conical tube and evaporated to dryness in a stream of nitrogen at 40°C. The residue was redissolved in 100 μl of mobile phase and then injected into the column.

Long-term stability

The long-term stability of MEX and its metabolites was studied after storing blank serum samples spiked with known amounts of these compounds for ten months at -25°C.

Preparation of calibration graph

Calibration graphs were constructed by the analysis of aliquots of drug-free serum spiked with a solution of MEX and its metabolites in a series of concentrations of 10–2000 ng/ml. The final concentration of the I.S. in each sample was 600 ng/ml. All samples were extracted and analysed as described. Peak-area ratios of MEX and each metabolite to the I.S. were calculated using six different concentrations of each compound analysed in triplicate. The data were then subjected to linear regression analysis to give the appropriate calibration factors.

Mass spectrometry

The HPLC eluent fractions corresponding to the retention times of MEX and its four metabolites were collected. The residues remaining after evaporation of the mobile phase were dissolved in 3 μl of methanol. Reconstituted samples were mixed briefly and 1.5-μl aliquots were then analysed by mass spectrometry (Finnigan MAT, Model 4000) using the direct inlet system. The identification was confirmed by comparing the spectra obtained with those of authentic standards. The data were processed by an Incos data system. The mass spectrometric operating conditions were: electron ionization energy, 70 eV; acceleration voltage, 2200 V; ion source temperature, 260°C. The mode of ionization was electron impact. The temperature of the solid probe was increased in a continuous manner from 25 to 250°C.

Application of the method

To test the applicability of this method for pharmacokinetic studies in patients, one healthy volunteer received an intravenous dose of 200 mg of mexiletine. The subject fasted overnight and did not eat on the day of study. Blood samples were drawn before and 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 18 and 24 h after administration of the drug. Serum was separated from the blood samples by centrifugation and frozen at -20°C until analysis.

Eleven serum samples from patients receiving long-term treatment with mexiletine were also analysed.

RESULTS AND DISCUSSION

Chromatographic separation

To optimize the chromatographic conditions

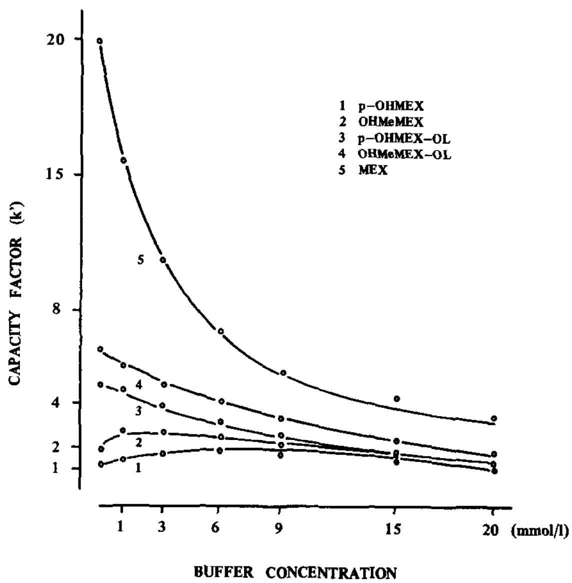

Fig. 2. Effect of buffer concentration on retention of MEX and its metabolites. Mobile phase, acetonitrile–methanol–water (120:40:334), pH 6.5; flow-rate, 1.2 ml/min.

for the separation of MEX and its four metabolites, the effect of the combination of organic modifiers, buffer composition and pH on the resolution of these compounds was investigated on a deactivated Supelcosil LC_8-DB column.

The Supelcosil LC_8-DB column was chosen after comparison with LiChrosorb RP-8 (Merck) and μBondapak C_{18} (Waters) columns which proved less satisfactory because of tailing effects.

Starting with acetonitrile–water (28:72, v/v) buffered with phosphate buffer, previously reported by Filipek *et al.* [22] and Selinger and Crawhall [25], resolution for the two main metabolites (*p*-OHMEX and OHMeMEX) was obtained, but their retention times were too short. However, *p*-OHMEX-OL and OHMeMEX-OL were not completely separated from one another. They could be resolved if either the percentage of the acetonitrile in the eluting solvent was decreased or if the flow-rate was decreased considerably. Unfortunately, these changes resulted in much longer retention times and excessive peak tailing with a subsequent loss of sensivity of MEX. Ternary solvent combinations were also used to try to separate these co-eluting peaks. It was finally found that using an acetonitrile–methanol–water (120:40:334, v/v) mobile phase provided the best resolution and highest efficiency.

Using this combination of organic modifiers, the influence of the phosphate buffer concentration on the retention of MEX and its metabolites was evaluated. It was found that the capacity factor (k') steadily diminished with increasing ionic strength, whereas a small increase was observed at low buffer concentrations for *p*-OHMEX and OHMeMEX (Fig. 2). The maximum resolution with symmetrical peaks in the shortest possible time was obtained using a buffer concentration of 9.0 mmol/l.

Of these chemical species, three (MEX, *p*-OHMEX and OHMeMEX) are weak amines, and their retention behavior is strongly influenced by the pH of the mobile phase. Decreasing the pH of the mobile phase to less than 4.5 increases the ionization of these compounds. These ionized forms are more soluble in the polar mobile phase and were less strongly retained on the non-polar stationary phase than the two remaining metabolites (*p*-OHMEX-OL and OH MeMEX-OL). A pH of 3.5 was chosen, which provided close to baseline separation of MEX and its four metabolites.

Under the chromatographic conditions used, the retention times of the peaks of interest were 4.12 (*p*-OHMEX), 4.92 (OHMeMEX), 6.29 (*p*-OHMEX-OL), 7.34 (OHMeMEX-OL), 11.1 (MEX) and 15.25 (I.S.) min.

The system was stable and reproducible, and during routine use the relative retention times of the compounds under study varied by only ±0.01.

Optimization of the extraction procedure

The sample preparation procedure was based on a widely used extraction technique. MEX, its metabolites and KOE 768, selected as the I.S., in serum samples could be extracted with hexane, dichloromethane, diisopropyl ether or a combination of these. The extraction was carried out under exactly the same conditions. The pH of the serum samples was adjusted to 9.5 prior to extraction with 200 μl of borate buffer solution. The extraction efficiency of the solvent tested was evaluated by comparing the peak areas obtained for identical compounds in the extracted samples to those in the standard mixture with the latter set as 1.0. The extraction with hexane showed low recoveries of all the compounds. However, the blank human serum extract yielded no interfering peaks from endogenous constituents. A double extraction procedure did not significantly increase the extraction efficiency. Dichloromethane, diisopropyl ether and their combination at various volume ratios gave a satisfactory extraction yield for MEX, *p*-OHMEX, OHMeMEX and the I.S., but the recoveries of the most polar metabolites (*p*-OHMEX-OL and OHMeMEX-OL) were insufficient. This problem was overcome when a small volume of propan-2-ol was added to the extraction mixture. Diisopropyl ether–dichloromethane–propan-2-ol (2.5:1.5:0.5, v/v) was found to be very suitable for extraction of the drug and its metabolites from the serum giving yields greater than 0.74 (Table I). This resulted in neglible interference in the chromatograms from the blank and spiked serum samples (Fig. 3A and B). When the serum samples from

TABLE I

EXTRACTION EFFICIENCY OF MEX, ITS METABOLITES AND THE I.S. USING DIFFERENT EXTRACTION SOLVENTS ($n=5$)

Compound	Hexane	Dichloromethane	Diisopropyl ether	Mixture I[a]	Mixture II[a]
MEX	0.56	0.71	0.98	0.97	0.85
OHMeMEX	0.72	0.86	0.75	0.75	0.96
p-OHMEX	0.69	0.77	0.82	0.78	0.82
p-OHMEX-OL	0.52	0.60	0.66	0.68	0.75
OHMeMEX-OL	0.76	0.62	0.64	0.65	0.88
I.S.	0.48	0.74	0.87	0.85	0.79

[a] Mixture I, diisopropyl ether–dichloromethane (4:1, v/v); mixture II, diisopropyl ether–dichloromethane–propan-2-ol (2.5:1.5:0.5, v/v).

patients were analysed using this extraction procedure, an extra peak of unknown origin was observed (Fig. 3C).

Confirmation by mass spectrometry

To confirm the HPLC results, the fractions corresponding to the retention times of MEX

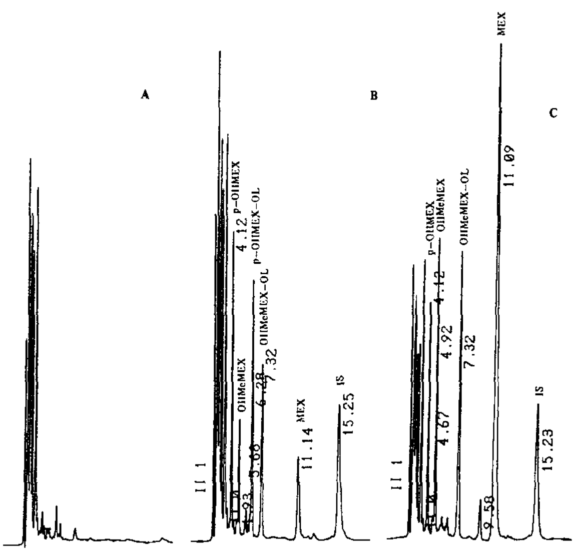

Fig. 3. Typical chromatograms obtained after extraction of (A) blank human serum, (B) blank human serum spiked with a mixture of MEX and its metabolites (100 ng) and (C) patient's serum containing about 95 ng of *p*-OHMEX, 346 ng of OHMeMEX, 180 ng of OHMeMEX-OL and 1240 ng of MEX. These samples were spiked with 300 ng of I.S. prior to extraction. Extraction mixture: diisopropyl ether–dichloromethane–propan-2-ol (2.5:1.5:0.5, v/v). Mobile phase, acetonitrile–methanol–water–phosphate buffer (120:40:334:6, v/v) adjusted to pH 3.5. Flow-rate 1.2 ml/min.

and its metabolites were collected and then analysed by mass spectrometry. Mass spectra from these fractions were compared with those of reference compounds. The mass spectra of MEX and its four metabolites from authentic standards are shown in Figs. 4–6. The electron-impact mass spectrum of MEX, *p*-OHMEX-OL and OHMeMEX-OL shows reasonably intense molecular ion peaks at m/z 179 and 196, respectively. However, no molecular ion peak was observed for the two main metabolites (*p*-OHMEX and OHMeMEX). The base peak of MEX, *p*-OHMEX and OHMeMEX is due to cleavage in the side-chain between carbon atoms giving ion m/z 44 or between carbon and oxygen atoms giving ion m/z 58. For the remaining metabolites, the same cleavage produces m/z 45 and 59 ions, in low abundance. The base peaks for these metabolites are characterized by m/z 138 and 120 ions, respectively. Other important characteristics ions of MEX include m/z 77, 91, 105, 107, 121 and 148. The mass spectra of the metabolites also contain the following low intensity ions: for OHMeMEX, m/z 77, 91, 105, 121, 137, 149 and 175; for *p*-OHMEX, m/z 69, 77, 91, 107, 123 and 149; for *p*-OHMEX-OL, m/z 69, 77, 91, 107, 109, 123 and 151; for OHMeMEX-OL, m/z 65, 77, 91, 105, 134, 149 and 152. The mass spectra of MEX, *p*-OHMEX and OHMeMEX are similar to those reported previously [26].

The electron-impact mass spectra of the collected HPLC fractions of MEX, *p*-OHMEX, OHMeMEX and OHMeMEX-OL were not sig-

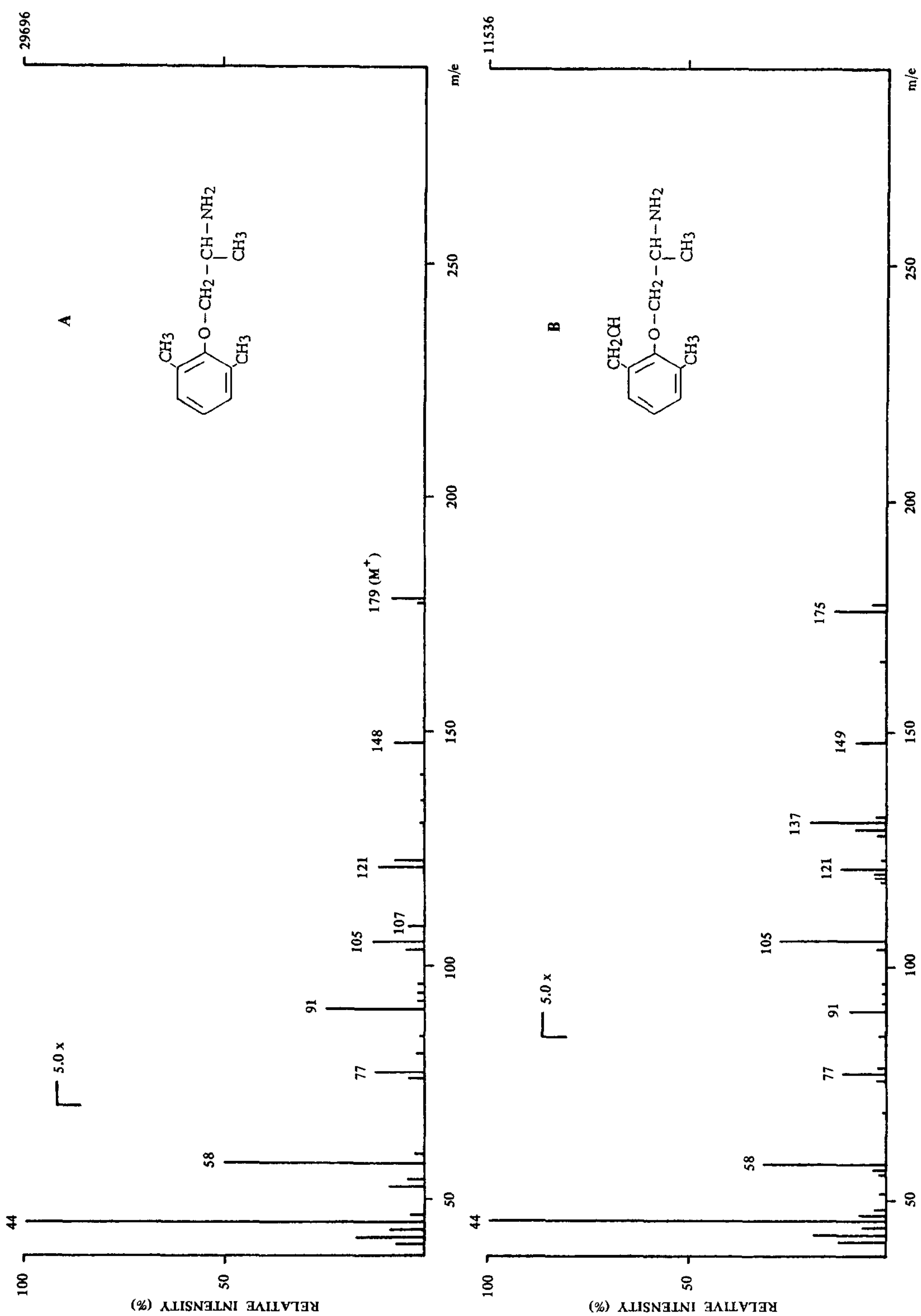

Fig. 4. Electron-impact mass spectra and structures of (A) MEX and (B) OHMeMEX from authentic standards.

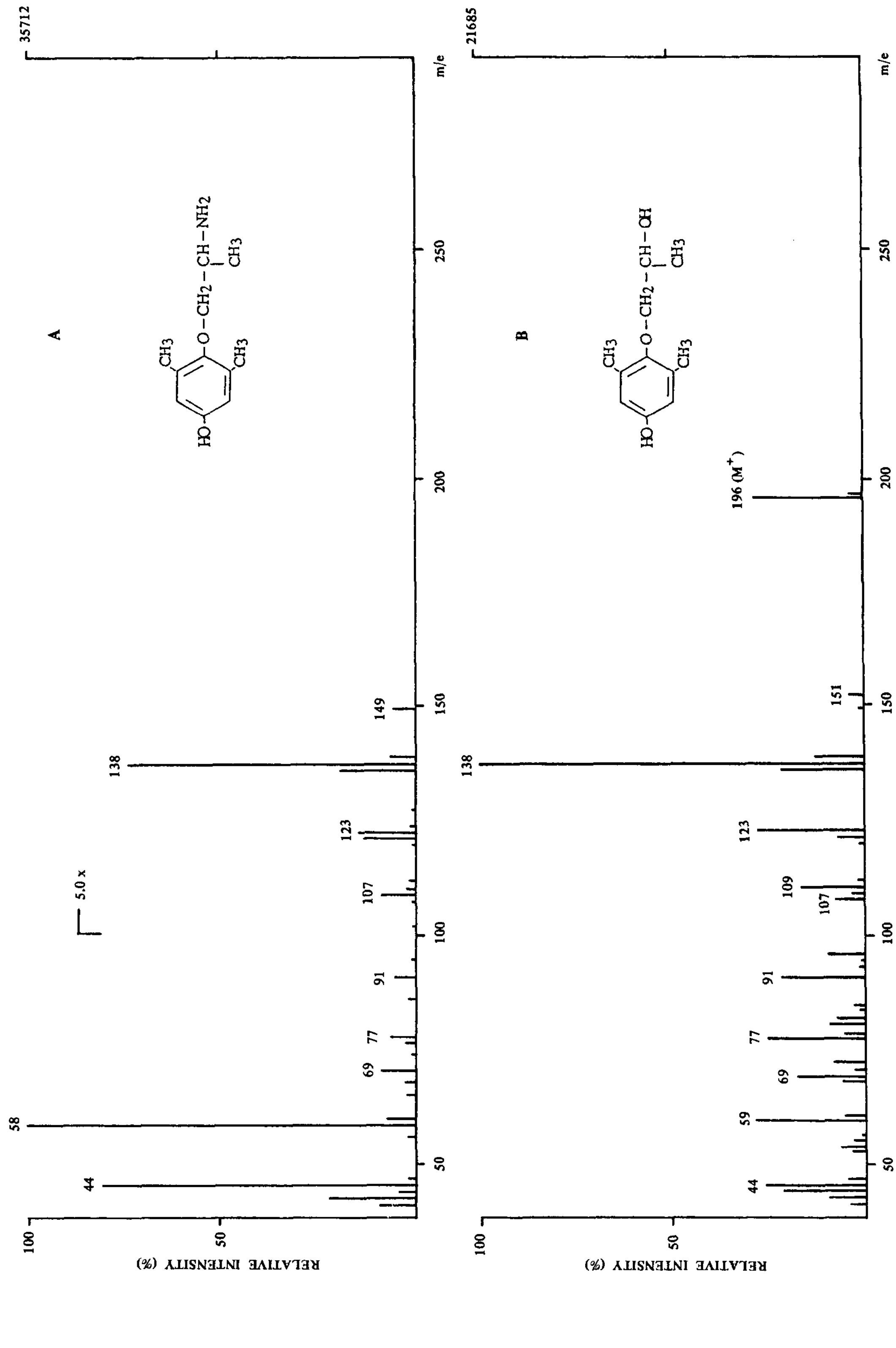

Fig. 5. Electron-impact mass spectra and structures of (A) *p*-OHMEX and (B) *p*-OHMEX-OL from authentic standards.

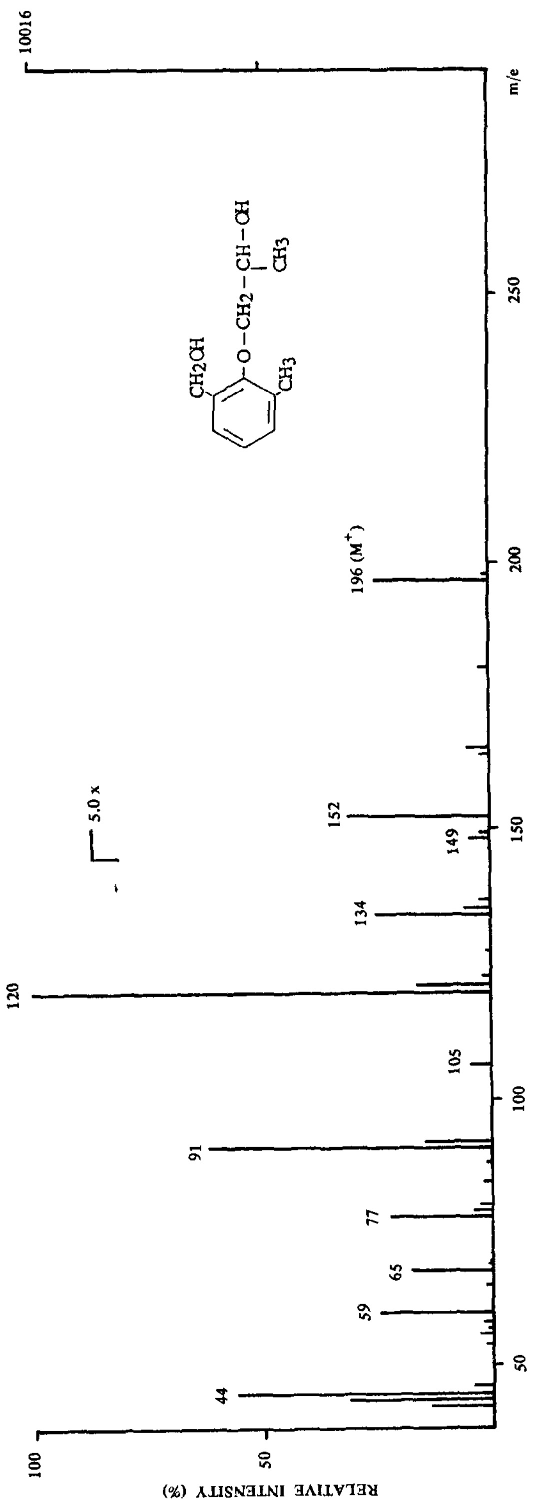

Fig. 6. Electron-impact mass spectrum of OHMeMEX-OL from authentic standard.

TABLE II

STABILITY OF MEX AND ITS METABOLITES IN FROZEN SERUM (−25°C)

Time (months)	Percentage of initial concentration [a]				
	MEX	OHMeMEX	*p*-OHMEX	OHMeMEX-OL	*p*-OHMEX-OL
1	99.4	100.2	99.6	98.7	99.6
2	99.5	99.8	99.3	99.0	99.2
4	99.7	99.4	98.8	99.2	99.0
7	99.3	99.1	99.2	99.0	98.6
10	99.2	99.0	98.9	98.9	98.7

[a] Serum samples spiked with 100 ng/ml of each compound.

nificantly different from the mass spectra of authentic standards. However, the mass spectrum of the fraction corresponding to *p*-OHMEX-OL confirmed the lack of this metabolite in the serum samples of patients.

Stability

Serum samples spiked with known amounts of MEX and its metabolites were stored in disposable polypropylene tubes at −25°C to assess their stability characteristics on storage. In all in-

TABLE III

PRECISION AND ACCURACY IN THE DETERMINATION OF MEX AND ITS METABOLITES IN SERUM ($n=3$)

Added (ng)	Intra-assay			Inter-assay		
	Recovered (mean ± S.D.) (ng)	C.V.[a] (%)	Accuracy (%)	Recovered (mean ± S.D.) (ng)	C.V.[a] (%)	Accuracy (%)
MEX						
10	9.0 ± 0.6	6.6	90.0	8.7 ± 0.8	9.1	87.0
100	95.4 ± 2.6	2.7	95.4	93.1 ± 3.3	3.5	93.1
1000	983.7 ± 29.7	3.0	98.3	977.2 ± 30.6	3.1	97.2
p-OHMEX						
10	9.3 ± 0.4	4.3	93.0	9.1 ± 0.4	4.3	91.0
100	97.5 ± 7.8	8.0	97.5	96.3 ± 9.0	9.3	96.3
1000	995.3 ± 11.7	1.1	99.5	987.0 ± 18.7	1.9	98.7
OHMeMEX						
10	9.6 ± 0.8	8.3	96.0	9.5 ± 0.8	8.4	95.0
100	99.6 ± 3.5	3.5	99.6	98.5 ± 4.1	8.3	98.5
1000	996.2 ± 9.3	0.9	99.6	992.3 ± 14.0	1.4	99.2
OHMeMEX-OL						
10	8.9 ± 0.7	7.8	89.0	8.7 ± 0.9	10.3	87.0
100	98.0 ± 8.6	8.7	98.0	97.6 ± 10.0	10.2	97.6
1000	986.3 ± 37.8	3.8	98.6	980.5 ± 53.2	5.4	98.0
p-OHMEX-OL						
10	9.3 ± 0.7	7.5	93.0	9.1 ± 0.8	8.7	91.0
100	101.2 ± 7.2	7.1	101.2	100.0 ± 6.0	6.0	100.0
1000	987.0 ± 27.0	2.5	98.7	980.0 ± 42.0	4.2	98.0

[a] C.V. = Coefficient of variation.

stances the concentrations of the compounds tested remained constant throughout the observation period (Table II).

Characteristics of the assay

The calibration graphs were obtained by analysing triplicate serum samples containing known amounts of MEX, its four metabolites and the I.S. Peak areas of MEX and its metabolites correlated linearly with their concentrations in the range tested (10–2000 ng/ml). Linear regression analysis of the data yields straight lines with an intercept of zero. The correlation coefficients of the lines constructed were 0.998. The limit of detection of the assay determined in extracted serum samples, defined as a signal-to-noise ratio of 4:1, was 2 ng/ml for all compounds. Inter- and intra-assay precision and accuracy were evaluated at three different concentrations of the parent drug and its metabolites in serum by analysing repeatedly within the same day and over a three-week period (Table III). The mean (±S.D.) coefficients of variation (C.V.) of MEX, *p*-OHMEX, OHMeMEX, OHMeMEX-OL and *p*-OHMEX-OL for intra-assay analysis were 4.1 ± 2.1, 4.4 ± 3.4, 4.2 ± 3.7, 6.7 ± 2.6 and 5.7 ± 2.7%, respectively, whereas for inter-assay analysis they were 5.2 ± 3.5, 5.1 ± 3.7, 6.0 ± 4.0, 8.6 ± 2.8 and 6.3 ± 2.2%.

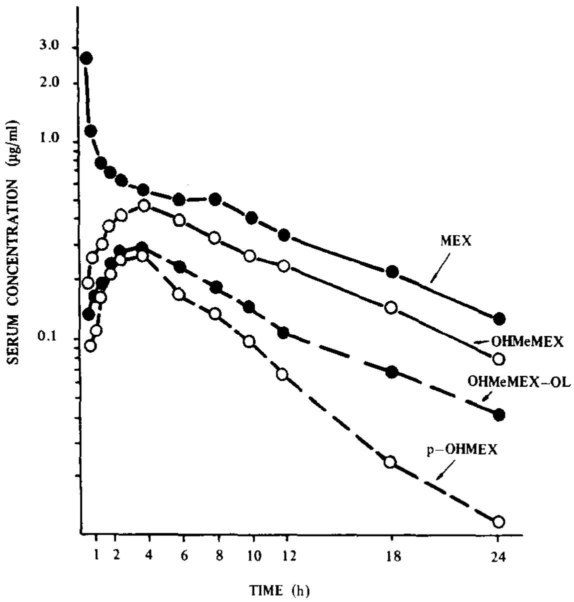

Fig. 7. Serum concentration *versus* time for MEX, *p*-OHMEX, OHMeMEX and OHMeMEX-OL in a healthy volunteer after intravenous injection of 200 mg of mexiletine.

Interferences

Interferences from other drugs and metabolites was evaluated by adding various compounds to serum samples in amounts representative of therapeutic or higher concentrations. These were then extracted and injected into the column. The following compounds were evaluated: digoxine,

TABLE IV

CONCENTRATIONS OF MEX AND ITS METABOLITES IN SERUM OF PATIENTS DURING LONG-TERM TREATMENT WITH MEX

Patient No.	Daily dose (mg)	Concentration (µg/ml)			
		MEX	*p*-OHMEX	OHMeMEX	OHMeMEX-OL
1	800	1.60	0.025	0.109	0.105
2	800	0.67	N.D.[a]	0.300	0.325
3	600	0.65	0.047	0.085	0.050
4	800	1.34	0.035	0.290	0.095
5	600	0.52	0.084	0.200	0.320
6	600	0.72	0.260	0.023	0.162
7	600	1.43	0.020	0.180	0.170
8	800	1.90	0.030	0.028	0.040
9	800	1.33	0.064	0.140	0.193
10	800	0.95	0.122	0.238	0.154
11	800	0.87	0.405	0.440	0.206

[a] Not detected.

disopyramide, N-desisopropyldisopiramide, quinidine, nitrazepam, sotalol, carvedilol, clobazam, N-desmethylclobazam, flecainide, propafenone, 5-hydroxypropafenone and propranolol. None of these compounds interfered with the determination of MEX and its metabolites. Other drugs such as amiodarone, desethylamiodarone, verapamil, norverapamil, gallopamil, prazosin, captopril, diltiazem, clonazepam, procainamide and N-acetylprocainamide were not detected.

Determination in serum samples from human subjects

The suitability of this method for the determination of MEX and its metabolites in serum from human subjects during single-dose pharmacokinetic studies is shown in Fig. 7. Following intravenous injection of 200 mg of MEX to a healthy volunteer the serum concentrations of *p*-OHMEX, OHMeMEX-OL, OHMeMEX and MEX were measurable over a 24-h period, whereas those of *p*-OHMEX-OL were not detected. The peak serum concentrations of *p*-OHMEX, OHMeMEX and OHMeMEX-OL were 0.267, 0.475 and 0.289 μg/ml, respectively. The estimated apparent serum elimination half-lives of MEX, *p*-OHMEX, OHMeMEX and OH MeMEX-OL were 8.9, 5.3, 8.3 and 8.5 h, respectively. The pharmacokinetic parameters of MEX and the two main metabolites, OHMeMEX and *p*-OHMEX, are comparable with those reported previously [5,6]. No pharmacokinetic parameters have been reported for OHMeMEX-OL.

Serum samples collected from eleven patients receiving mexiletine by mouth were also analysed (Table IV). These samples had no detectable level of *p*-OHMEX-OL. Mass spectrometric analysis of these samples was also performed.

CONCLUSIONS

This HPLC method using a deactivated LC_8-DB column under isocratic conditions gives excellent separation of the drug, its metabolites and the I.S., with a reasonable analysis time and appropriate sensitivity for detection during single-dose pharmacokinetic studies. The method described is simple and rapid as it requires only a single extraction step prior to injection.

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