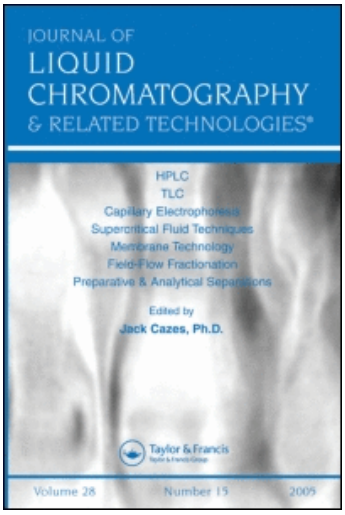

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Fluorescence Detection of Mexiletine and Its *P*-Hydroxylated and Hydroxy-Methylated Metabolites in Human Plasma and Urine by High-Performance Liquid Chromatography Using Post-Column Derivatization with *O*-Phthalaldehyde

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FLUORESCENCE DETECTION OF MEXILETINE AND ITS *p*-HYDROXYLATED AND HYDROXY-METHYLATED METABOLITES IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING POST-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE

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ABSTRACT

A liquid chromatographic procedure for the simultaneous determination of mexiletine and two major metabolites, *p*-hydroxymexiletine and hydroxymethylmexiletine, in plasma and urine is described. A reversed-phase ion-pair C_{18} column is used with gradient elution using post-column fluorescence derivatization with o-phthalaldehyde. The lower limits of detection are 2 ng/ml for mexiletine and *p*-hydroxymexiletine and 5 ng/ml for hydroxymethylmexiletine. Enzymatic hydrolysis of conjugate forms of oxidative metabolites in urine samples was also performed. The coefficients of variation for replicate assay of spiked samples were uniformly less than 10 % for all the analytes.

INTRODUCTION

Mexiletine (1-(2',6'-dimethylphenoxy)-2-aminoproprane) is a class 1B antiarrhythmic agent used for the control of ventricular arrhythmias (1). Mexiletine is eliminated mainly by hepatic oxidation (2). Among several oxidative metabolites, *p*-hydroxymexiletine and

hydroxymethylmexiletine are considered major metabolites (2,3), and aromatic hydroxylation to *p*-hydroxymexiletine and aliphatic hydroxylation to hydroxymethylmexiletine co-segregates with that of debrisoquine or sparteine (4,5). The oxidation of mexiletine and the formation of its metabolites are quite different between extensive and poor metabolizers of debrisoquine (5) . Although many methods have been reported to detect mexiletine in plasma or serum using HPLC (6,7,8,9,10), a few methods are available to determine mexiletine and *p*-hydroxymexiletine and hydroxymethylmexiletine simultaneously (11,12).

o-Phthalaldehyde (OPTA) has been widely used for the fluorometric analysis of amino acids and biologically active amines (13,14). OPTA easily reacts with primary amines in alkaline medium in the presence of 2-mercaptoethanol, and forms strongly fluorescent isoindolic derivatives. The pre-column method with an OPTA reagent was reported to detect mexiletine and tocainide in plasma (15).

The aim of this study was to develop an HPLC assay for simultaneous determination of mexiletine and its main metabolites, *p*-hydroxymexiletine and hydroxymethylmexiletine, following the formation of a post-column derivative with OPTA in plasma and urine.

MATERIALS and METHODS

Chemical and reagents

Mexiletine, *p*-hydroxymexiletine, hydroxymethyomexiletine and 4-methylmexiletine (internal standard, IS) were generously provided by Nippon Boehringer Ingelheim (Hyo-go, Japan). 1-Octanesulfonic acid sodium salt (HPLC grade) was obtained from Kodak (Rochester, NY, USA). OPTA and mercaptoethanol were purchased from Tokyo

Chemical (Tokyo, Japan) and Wako (Osaka, Japan), respectively. β-Glucuronidase extracted from Helix pomatia (Type H-5) was obtained from Sigma (St.Louis, MO, USA). All other reagents were of analytical-reagent grade. Deionized and distilled water was used throughout this investigation.

Preparation of standards

Stock standard solutions of mexiletine, *p*-hydroxymexiletine, hydroxy-methylmexiletine and 4-methylmexiletine were prepared by dissolving 50 mg of each compound in 50 ml of methanol and stored at 4 °C. Plasma standards for calibration or precision determination in the range 0.01-1.0 μg/ml were prepared by diluting these stock solutions with fresh plasma obtained non-medicated normal volunteers.

Instrumentation and conditions

The chromatographic system consisted of two pumps (880PU, Jasco, Tokyo, Japan), a solvent mixing module (880-31, Jasco, Tokyo, Japan), an automatic injector (855AS, Jasco, Tokyo, Japan), a Wakosil ODS Column (4.6 mm I.D. x 25 cm, Wako, Osaka, Japan) protected with a Wakosil ODS guard column (4.6 mm I.D. x 1 cm, Wako, Osaka, Japan), fluorometer (821FP, Jasco, Tokyo, Japan) and a chart recorder (Chromatocorder12, SIC, Tokyo, Japan). For the post-column method, the column eluate was mixed with OPTA reagent which was delivered by a pump (880PU, Jasco, Tokyo, Japan) through a T-piece (851R, Jasco, Tokyo, Japan). The mixture were allowed to flow successively through 0.5 mm x 5 m long tefzel-tube (865R, Jasco, Tokyo, Japan). The

column with the guard column and tefzel-tube were in a column oven (Oven A-30, Shodex, Tokyo, Japan).

The mobile phase was a mixture of the mobile phase "A" and "B", which consisted of 10 mM H_3PO_4, methanol and 1.4 mM 1-Octanesulfonic acid, the proportions of which were controlled by a gradient program. The mobile phase was delivered at a constant flow rate of 1.0 ml/min. The mobile phase "A" was prepared by mixing 160 ml of methanol, 840 ml of 10 mM H_3PO_4 and 300 mg of 1-Octansulfonic acid sodium salt. The mobile phase "B" was prepared by mixing 800 ml of methanol, 200 ml of 10mM H_3PO_4 and 300 mg of 1-Octansulfonic acid sodium salt. The gradient was 0 to 10 min:65 to 20 % A, 10 to 14 min:20 % A, 14 to 15 min:20 to 65 % A, 15 to 25 min: 65 % A.

The OPTA reagent was prepared everyday by mixing 0.5 ml of mercaptoethanol, 900 ml of 0.2 M borate buffer (PH 10.0) and 100 ml of ethanol dissolved 300 mg of OPTA in advance. This reagent was delivered at a flow-rate of 0.6 ml/min.

The fluorometer was set to an excitation wavelength at 345 nm and an emission wavelength at 445 nm.

Extraction procedure

A 1 ml aliquot of plasma was introduced into a 10ml capped polypropylene centrifuge tube to which 50 ml of the I.S. solution (1 mg), 1 ml of 0.2 M borate buffer (pH 9) and 7 ml of diethyl ether were added. After the mixture was agitated on a reciprocating shaker for 10 min and then centrifuged at 3000 g for 5 min, the sample was frozen in a methanol-dry ice bath. The organic phase was transferred into another polypropylene tube and evaporated to dryness in a stream of nitrogen at 40 °C. The residue was reconstituted with 250 μl of 50:50 mixture of the mobile phase "A" and "B" for a second and 100 μl were injected on to the column.

Enzymatic hydrolysis of conjugate metabolites in urine

A 100 μl aliquot of urine was introduced into a 10ml capped polypropylene centrifuge tube to which 300 μl of 1 M sodium acetate buffer (pH 5.5) and 5.7 mg of Sigma Type H-5 enzyme, containing 440,000 U/g β-glucuronidase (i.e., 2500 U) were added. After vortexing, the tubes were incubated for 1, 2, 3, 4, 6 and 10 hours at 37 °C in a water bath. Following hydrolysis, the samples were mixed with 100 μl of the I.S. solution (2 μg), 1 ml of 0.2 M borate buffer (pH 10) and 7 ml of diethyl ether. Other extraction procedure was same as plasma.

Calibration graph and Recovery

Calibration graphs for mexiletine, *p*-hydroxymexiletine, hydroxymethymexiletine were constructed by the analysis of three standard solutions with the concentrations 0.01, 0.1, 1.0 μg/ml for each compound. Linear calibration plots of mexiletine and its metabolites were generated by least-squares regression of the peak-area ratios of the analyte to the internal standard. The recovery was determined by comparing the peak areas of mexiletine and its metabolites obtained by analyzing a standard plasma sample with the peak areas obtained by direct injection of the standard solution.

Application of the method

Two fully informed healthy subjects received 150 mg of mexiletine orally after overnight fasting. Blood samples were obtained just before and at 0.5, 1, 2, 4, 6, 9, 24, 33 and 48 hours after dosage. Blood samples were mixed with a small amount of heparin

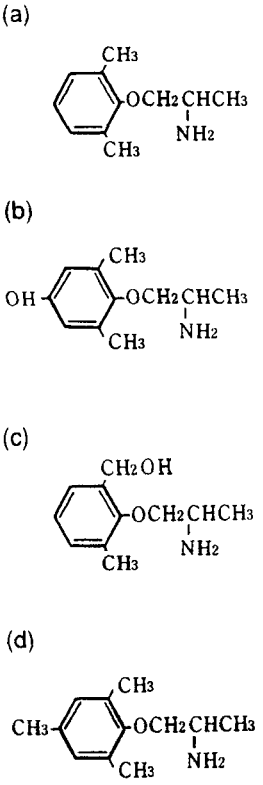

FIGURE 1. Structure of (a) mexiletine, (b) *p*-hydroxymexiletine, (c) hydroxymethylmexiletine, and (d) the internal standard.

and immediately centrifuged at 3000 g, and plasma was stored at - 20 °C until analyzed. Urine was collected for 48 hours and was stored in a refrigerated glass tube at - 20 °C.

RESULTS and DISCUSSION

Recently OPTA has been widely used as a pre-column and post-column derivation reagent for the fluorometric analysis of compounds containing a primary amines. Mexiletine, *p*-hydroxymexiletine, hydroxymethylmexiletine and 4-methylmexiletine contain

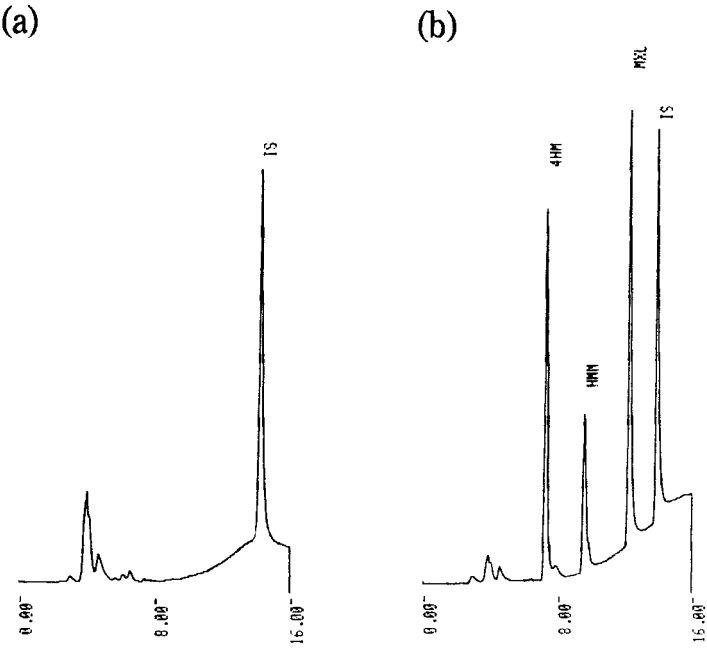

FIGURE 2. Chromatograms obtained from (a) blank plasma containing 1 μg the internal standard, and (b) standard sample containing 1 μg each of *p*-hydroxymexiletine (HM), hydroxymethylmexiletine (HMM), mexiletine (MXL) and the internal standard (IS).

a primary amine group at the β-position of the carbon chain (Figure 1). Although pre-column derivation is convenient for the detection of mexiletine, there were many interfering peaks in a chromatogram of blank plasma and besides, in our experiences, the peaks of *p*-hydroxymexiletine and hydroxymethylmexiletine could not be separated from each other. Therefore, we planned to separate mexiletine and its metabolites by reversed-phase ion-pair HPLC with gradient elution and then to combine them with OPTA.

Typical chromatogram for the extracts of the blank plasma with the I.S. and standard plasma are shown in Figure 2. In post-column derivation, there is no interfering peak to detect mexiletine, its metabolites and the I.S..

TABLE 1.

Coefficients of variation and recovery of *p*-hydroxymexiletine, hydroxymethylmexiletine and mexiletine.

	Intra-assay	Inter-assay	recovery
	n=6	n=7	n=3
p-hydroxymexiletine			
10 ng	6.9 %	9.0 %	96.4 %
100 ng	4.6 %	5.5 %	93.0 %
1000 ng	3.5 %	6.0 %	89.3 %
hydroxymethylmexiletine			
10 ng	7.4 %	9.7 %	73.9 %
100 ng	6.2 %	7.9 %	56.9 %
1000 ng	4.7 %	7.6 %	52.2 %
mexiletine			
10 ng	6.3 %	8.2 %	96.4 %
100 ng	3.4 %	5.6 %	93.1 %
1000 ng	2.9 %	4.4 %	88.6 %

The calibration graphs were obtained by analyzing plasma samples at three concentration of 0.01, 0.1 and 1.0 μg/ml. The ratio of peak areas of mexiletine and its metabolites to the I.S. correlated linearly with their concentrations. The correlation coefficients of the lines constructed were 0.998, 0.997 and 0.998 for p-hydroxymexiletine, hydroxymethylmexiletine and mexiletine, respectively. The limit of detection of the assay

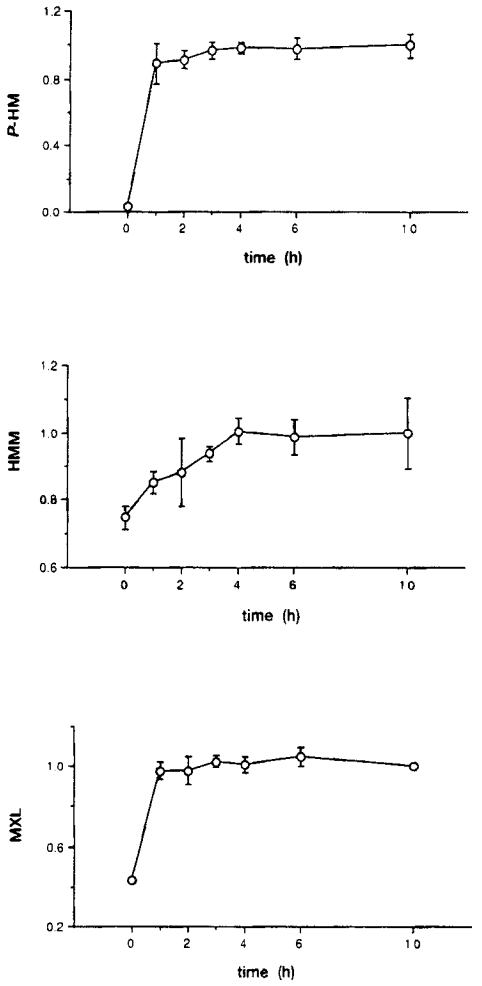

FIGURE 3. Effect of incubation time on the ratio of hydrolysis of *p*-hydroxymexiletine, hydroxymethylmexiletine, and mexiletine in a 100 μl of No 1 subject urine with 2500 U of β-glucuronidase. n=6, mean ± SD.

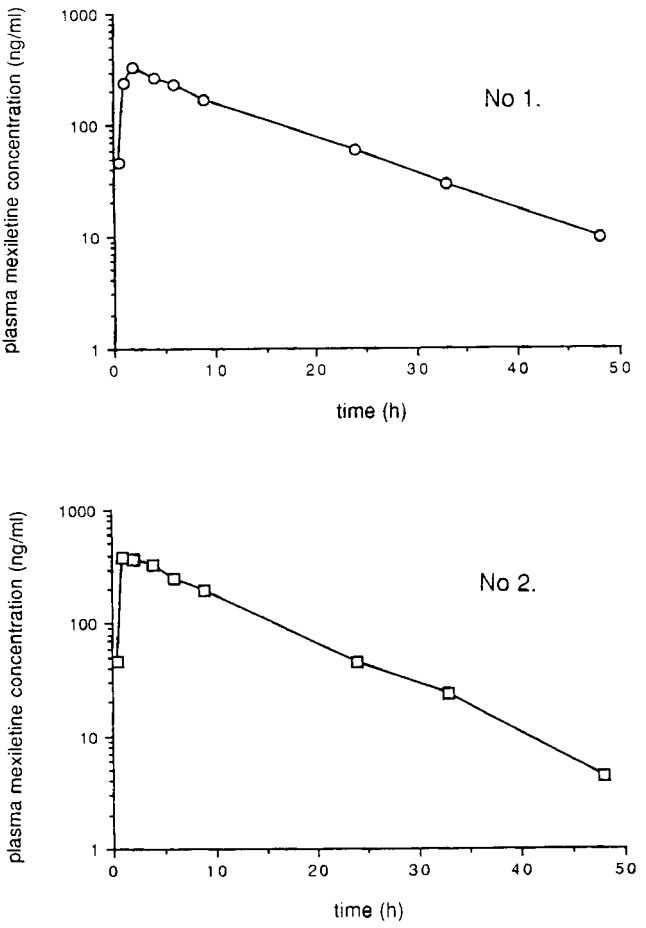

FIGURE 4. Plasma concentration time curve for mexiletine in two healthy volunteer after oral administration of 150 mg mexiletine hydrochloride.

determined in extracted plasma samples was 2, 5, and 2 ng/ml for *p*-hydroxymexiletine, hydroxymethylmexiletine and mexiletine, respectively. Table 1 shows the coefficient of variations of mexiletine and its metabolites for intra-assay and inter-assay and their recoveries. Diethyl ether extraction yielded an satisfactory extraction for *p*-hydroxymexiletine and mexiletine, whereas the recovery of hydroxymethylmexiletine

TABLE 2.

Pharmacokinetics and urinary recovery for 48 hours of mexiletine and its metabolites after a single oral administration of 150 mg mexiletine hydrochloride in two healthy subjects.

		No 1	No 2
Pharmacokinetics			
	Cmax (ng/ml)	327.2	377.9
	tmax (h)	2	1
	AUC (ng×h/ml)	4448.7	4690.0
	t1/2 (h)	9.3	7.3
	CLtot (L/h)	28.0	26.6
	CLren (L/h)	5.8	3.2
	CLhep (L/h)	22.2	23.3
Urinary recovery			
	mexiletine (%)	8.9	3.7
	p-HM (%)	22.0	20.1
	HMM (%)	19.9	26.1
	n-HM (%)	9.3	12.8

Cmax:maximum concentration, tmax:time of Cmax, AUC:area under the concentration -time curve from 0 to infinity. t1/2:terminal half-life, CLtot: apparent total clearance, CLren:renal clearance, CLhep:hepatic clearance (CLtot-CLren).
Values in urinary recovery are expressed as % of the administrated dose.
p-HM:*p*-hydroxymexiletine, HMM:hydroxymethylmexiletine, *n*-HM:*n*-hydroxymexiletine.

was insufficient. Since the coefficient of variation of hydroxymethylmexiletine was small, this extraction procedure was applied.

p-Hydroxymexiletine and hydroxymethylmexiletine are eliminated in urine unchanged and as glucuronide conjugates. *n*-Hydroxymexiletine is also eliminated in urine as a glucuronide conjugate. But this conjugate is reported to be changed to mexiletine after hydrolysis(5). To determine the incubation time, the concentrations of *p*-hydroxymexiletine, hydroxymethylmexiletine and mexiletine from a 100 μl of urine collected for 48 hrs from No. 1 subject were measured after incubation for 1, 2, 3, 4, 6 and 10 hours with 2500 U of β-glucuronidase. Incubations were carried out at 37 °C. Figure 3 shows that the percent of concentrations of three compounds after incubation for each period, compared with the concentrations after incubation for 10 hours. Maximal yield of the hydrolyzed metabolites and mexiletine were attained within 4 hours. The coefficients of variation of intra-day assay after enzymatic hydrolysis of urine samples (n=10) were 3.6 %, 3.5 % and 2.7 % for *p*-hydroxymexiletine, hydroxymethylmexiletine and mexiletine (*n*-hydroxymexiletine), respectively. Table 2 shows the pharmacokinetic parameters of mexiletine and the fractionary recovery of its metabolites from two subjects after the oral administration of 150 mg mexiletine.

In conclusion, the present post-column detection method shows good reproducibility and sensitivity for the determination of mexiletine and its metabolites in plasma and urine. This assay method can be recommended for pharmacokinetic study, especially in determining metabolic clearance.

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