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Fluorimetric determination of mexiletine in serum by high-performance liquid chromatography using pre-column derivatization with fluorescamine

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ABSTRACT

A simple, specific and sensitive micro-scale method for the assay of the antiarrhythmic agent mexiletine in human serum is described. The method uses high-performance liquid chromatography, with pre-column fluorimetric derivatization by fluorescamine. Following extraction with diethyl ether, mexiletine and 4-methylmexiletine (an internal standard) were derivatized with fluorescamine under weakly alkaline condition (pH 9.0) and chromatographed on a reversed-phase column with aqueous methanol–2-propanol as the mobile phase. The two fluorescent derivatives of mexiletine and the internal standard were separated as clear single peaks, and no interfering peaks were observed on the chromatograms. The detection limit for mexiletine was 0.005 μg/ml from only 100 μl of serum, and the calibration curves in the range 0.01–5 μg/ml were linear, with an overall coefficient of variation of less than 5%. The analytical recovery of a known amount of mexiletine added to serum was almost 100%. This method proved to be effective in the rapid monitoring of the serum concentrations in patiens who received this potent antiarrhythmic drug.

INTRODUCTION

Mexiletine is an antiarrhythmic drug, which belongs to Class IB according to Williams' classification and is widely used for the treatment of ventricular arrhythmias [1–3]. The therapeutic range of the serum concentration of mexiletine has been reported to be 0.5–2.0 μg/ml, whereas toxicity occurs above 2.0 μg/ml [4–5] and tremors frequently appears above 1.3 μg/ml [6]. In addition to the narrow therapeutic range, the pharmacokinetics of mexiletine are highly dependent on the condition of the patient and/or the existence of other effective coadministered drugs [7,8]. Therefore, to maintain an optimal antiarrhythmic effect without toxicity, routine monitoring of serum mexiletine is required.

Recently, several methods have been developed for the determination of mexiletine in plasma or serum. Some of these are gas chromatographic methods, which require flame ionization detection [9–13], electron-capture detection [14,15] or nitrogen-selective detection [16–19]. Other workers have employed UV ab-

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sorption detection with high-performance liquid chromatography (HPLC) [20,21].

This report describes a novel micro-scale method for the rapid analysis of serum mexiletine by HPLC, using pre-column derivatization with fluorescamine.

EXPERIMENTAL

Materials

Mexiletine and 4-methylmexiletine (internal standard, I.S.) were kindly supplied by Nippon Boehringer Ingelheim (Hyougo, Japan). Fluorescamine (Fluram, purity *ca.* 99%) was purchased from Nacalai Tesque (Kyoto, Japan). Ethanol and 2-propanol were of liquid chromatographic grade, and deionized, distilled water was used throughout this investigation. All other chemicals were of reagent grade and were used without further purification.

Preparation of standards

Stock solutions of mexiletine (1–500 μg/ml) and the I.S. solution (10 μg/ml) were prepared monthly in distilled water and stored in the dark at 4°C. Serum standards for calibration or precision determination in the range 0.01–5.0 μg/ml were prepared by diluting these stock solutions with fresh serum obtained from non-medicated normal volunteers.

Apparatus and chromatographic conditions

The chromatographic assembly consisted of a Model LC-3A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Model 7125 syringe loading injector (Rheodyne, Cotati, CA, U.S.A.) and a Model RF-510 spectrofluorophotometer (Shimadzu). The column was a reversed-phase column, Shim-Pack CLC-ODS(M) (particle size 5 μm, 250 mm × 4.6 mm I.D., Shimadzu). The column temperature was maintained at 60°C by a Model CTO-2A temperature-control module (Shimadzu). The mobile phase consisted of 55% 0.1 *M* tris(hydroxymethyl)aminomethane (adjusted to pH 9.0 with hydrochloric acid), 35% ethanol and 10% 2-propanol. The solvent was filtered and degassed before use. The flow-rate of the mobile phase was set at 1.0 ml/min, and the column pressure reached 150 bar. The column effluent was monitored at 397 nm (excitation wavelength) and 479 nm (emission wavelength). The quantitation of mexiletine was calculated using the peak-area ratio compared with the I.S.

Sample preparation

After 100 μl of serum were pipetted into a 10-ml screw-capped glass centrifuge tube, 20 μl of the I.S. solution (10 μg/ml in distilled water) were added and then vortex-mixed for a few seconds. Then 1 ml of sodium borate buffer (pH 9.0) and 7 ml of diethyl ether were added, and the mixture was agitated on a reciprocating shaker for 10 min and then centrifuged at 2500 *g* for 5 min at 4°C. The upper

organic layer was transferred to another glass tube and evaporated under reduced pressure at room temperature. The residue was vortex-mixed with 70 μl of sodium borate buffer (pH 9.0) for a few seconds and then vortex-mixed with 30 μl of fluorescamine in dioxane (0.15 mg/ml) for a few seconds. A 90-μl sample of the resultant mixture was injected into the HPLC column within 30 min.

The within-run precision of the assay was determined by adding mexiletine at three different concentrations to pooled normal human serum and assaying ten sample aliquots in the same assay run. The between-run precision was determined by assaying aliquots of these samples on ten consecutive days. Analytical recoveries of the assay were determined by adding mexiletine at appropriate clinical concentrations to serum from patients who received mexiletine orally.

Pharmacokinetic study

Two fully informed patients who had WPW syndrome received an intravenous administration of mexiletine. Blood samples were withdrawn into blood-collecting tubes at 0.25, 0.5, 1, 2, 3, 6, 12 and 24 h after administration without anticoagulant. The blood samples were centrifuged at 800 *g*, and the serum was removed and kept at -20°C until analysis. Serum concentrations were fitted to the following biexponential equation with a microcomputer-based non-linear regression program [22]: $C(t) = A\exp(-\alpha t) + B\exp(-\beta t)$, where $C(t)$ is the serum concentration of mexiletine at time t, A and B are zero time intercepts, and α and β are disposition rate constants. The pharmacokinetic parameters were calculated according to the following equations: $AUC^{0-\infty} = A/\alpha + B/\beta$, V_d = dose/$AUC^{0-\infty}/\beta$ and $t_{1/2\beta} = 0.693/\beta$, where $AUC^{0-\infty}$ is the area under the curve, V_d is the apparent volume of distribution and $t_{1/2\beta}$ is the elimination half-life.

RESULTS AND DISCUSSION

The derivatizing agent, fluorescamine, reacts directly with primary amines to form the same fluorophores at room temperature, and is widely used for the detection of amino acids and peptides of low molecular mass [23]. Mexiletine and 4-methylmexiletine contain a primary amine group at the β-position of the carbon chain (Fig. 1). Excitation and emission spectra of the fluorescent compounds formed from the reaction of mexiletine and 4-methylmexiletine with fluorescamine are shown in Fig. 2a. These fluorescent compounds show a maximum at 397

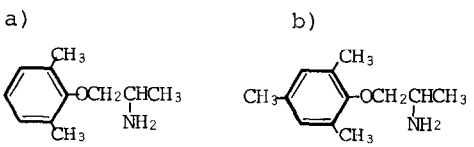

Fig. 1. Structures of (a) mexiletine and (b) 4-methylmexiletine. These compounds have a primary amine group at the β-position of the carbon chain.

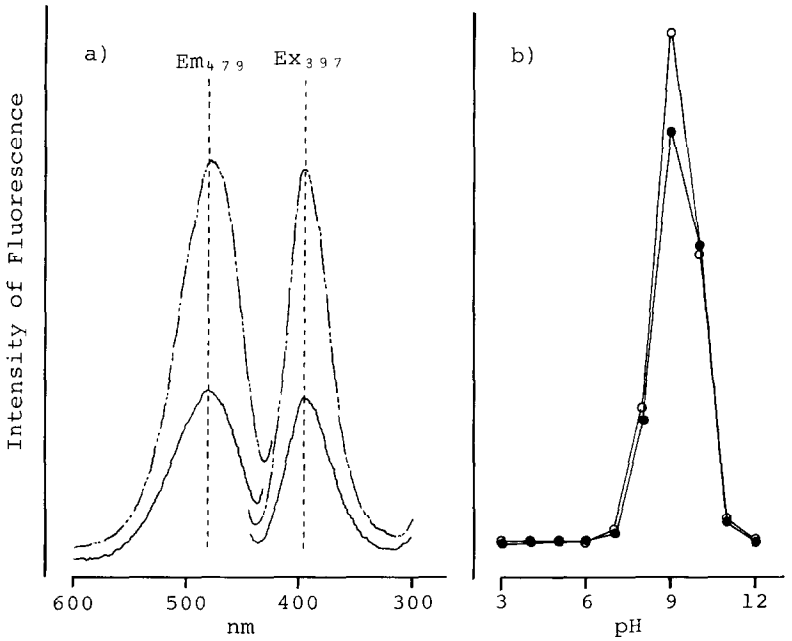

Fig. 2. Excitation and emission spectra of fluorescent compounds, and the effect of pH on the intensity of fluorescence in a test-tube. (a) Drugs were diluted with 3 ml of sodium borate buffer (pH 9.0) at a concentration of 1 μg/ml for mexiletine (———) and 2 μg/ml for 4-methylmexiletine (----), then 100 μl of fluorescamine in dioxane solution (0.15 mg/ml) were added to each sample. (b) Drugs were diluted with 3 ml of McIlvain buffer (pH 3–7), sodium borate buffer (pH 8–9) or sodium hydroxide solution (pH 10–12) at a concentration of 1 μg/ml, then 100 μl of fluorescamine in dioxane solution (0.15 mg/ml) were added to each sample. Key: mexiletine (●); 4-methylmexiletine (○).

for excitation and at 479 nm for emission. Fig. 2b shows the fluorescence intensity of the fluorescent compounds at various pH values in a test-tube: the maximum intensity was found at pH 9.0. At this pH, the reaction proceeds instantaneously at room temperature. Excess reagent is concomitantly destroyed with a half-life of several seconds, and neither fluorescamine nor its hydrolysis products are fluorescent [24].

Typical chromatograms for the extracts of the blank and standard serum, and serum from patients who received mexiletine orally are shown in Fig. 3. For the drug-free serum, no fluorescence could be detected on the chromatograms (Fig. 3a). The retention times for the fluorescamine derivatives of mexiletine and the I.S. were 14.0 and 20.0 min, respectively, and each fluorescamine derivative was separated as a clear single peak (Fig. 3b, c and d). As for the extract of hemolysed serum from a patient, no interfering peaks were detected at the retention times of mexiletine and the I.S. (Fig. 3d). From the results of a routine assay using 103 serum samples obtained from patients who received oral doses of mexiletine, no interfering peaks due to other coadministered drugs were found in serum of

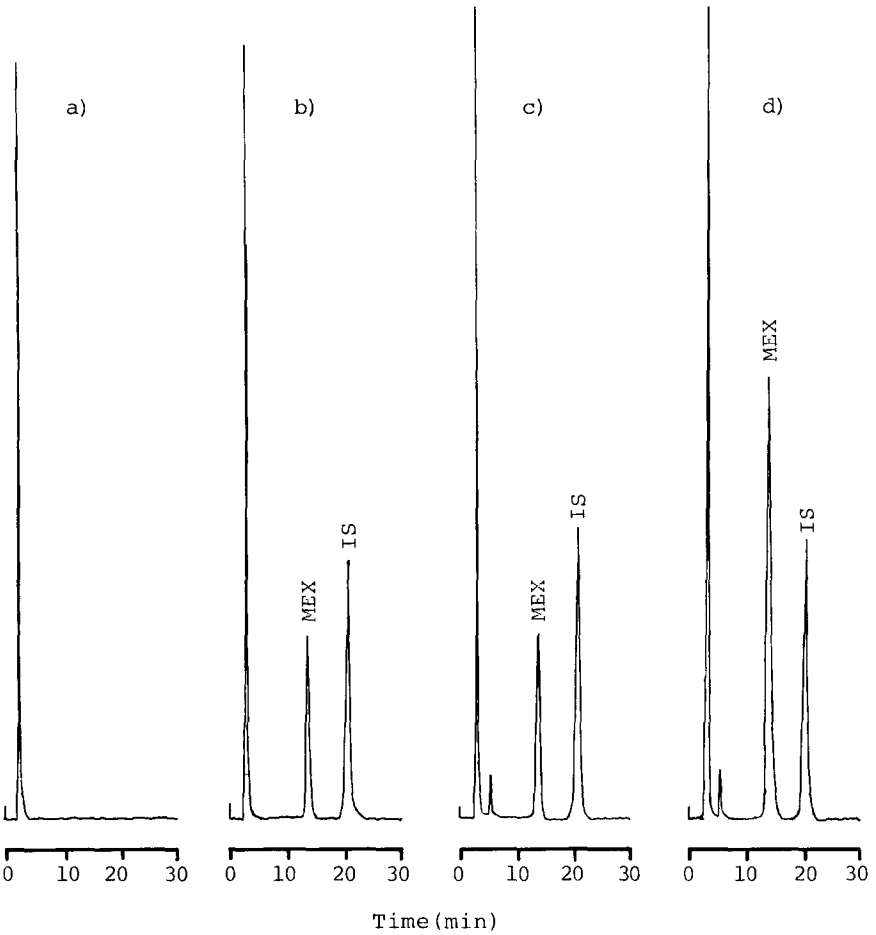

Fig. 3. Typical chromatograms of serum samples: (a) chromatogram of a blank serum sample from a normal subject; (b) chromatogram of a serum sample for the calibration at a concentration of 1 μg/ml mexiletine; (c) chromatogram of a serum sample from a patient; (d) chromatogram of a hemolysed serum sample from a patient. Peaks: MEX = mexiletine; IS = 4-methylmexiletine.

patients who received the following drugs: isosorbide dinitrate, nitroglycerin, amylnitrite, diltiazem, verapamil, nicardipine, disopyramide, nicorandil, dipyridamole, quinidine, nifedipine, ticlopidine, digoxin, digitoxin, dilazep, trapidil, trimetazidine, isoproterenol, lidocaine, acebutolol, penbutolol, trichlormethiazide, furosemide, triamterene, aminophylline, diazepam, nitrazepam, triazoram, salicylate, ubidecarenone, heparin, allopurinol and warfarin. The accuracy and precision of the method were determined by estimating the coefficient of variation (C.V.) of mexiletine measurements using ten replicated samples at concentrations of 0.5, 2 and 5 μg/ml. The within-run C.V. calculated for samples at three concentrations were 3.1, 2.6 and 2.1%, respectively, and the between-run C.V. were 4.9,

3.0 and 2.5%, respectively. When the peak-area ratios of mexiletine to the I.S. were plotted against the concentration of plasma samples spiked with mexiletine over the concentration range 0.01–5.0 μg/ml, the resultant standard curves were linear and passed through the origin with a correlation coefficient of 0.997 or better, and the limit of detection for mexiletine was 0.005 μg/ml. To check the efficiency and reproducibility of the extraction procedure, analytical recoveries from patients' serum samples with added mexiletine at three concentrations were obtained. The mean recovery obtained was almost 100% (Table I).

Diethyl ether extraction of serum samples in the concentration range 0.01–5.0 μg/ml yielded an absolute recovery of 91–93% for mexiletine and 95–98% for 4-methylmexiletine. No serum proteins were detected by the method of Lowry *et al.* [25] in the residue after evaporation. This method provided rapid extraction of mexiletine and the I.S. As for the mobile phase, optimal separation of fluorescamine derivatives of mexiletine and 4-methylmexiletine from endogenous constituents of serum was obtained by adding 0.1 *M* Tris as the modifier. It improved the solvent selectivity and provided sharp, symmetrical and well defined peaks of derivatized mexiletine and the I.S.

It is well known that there are two main metabolites of mexiletine (4-hydroxymexiletine and 2-hydroxymethylmexiletine) found in serum or urine [12]. These metabolites also have primary amine groups at the β-position of the carbon chain and are able to react with fluorescamine, however, no interfering peaks due to fluorescamine metabolite derivatives were observed on the chromatograms. Since the polarity of the metabolites is generally larger than that of the parent drug, fluorescamine derivatives of these metabolites may have eluted within 10 min under the chromatographic conditions employed here. However, this procedure appears to be potentially applicable to the determination of mexiletine metabolites with a minor modification in the analytical conditions of HPLC. This extrac-

TABLE I

RECOVERY OF MEXILETINE ADDED TO PATIENTS' SERUM USING FLUORIMETRIC HPLC

Sample	Concentration (μg/ml)			Recovery (%)
	Initial	Added	Found	
A	0.232	0.1	0.333	101.0
		1.0	1.232	100.0
		3.0	3.294	102.1
B	0.762	0.1	0.867	105.0
		1.0	1.764	100.2
		3.0	3.732	99.0
C	1.983	0.1	2.085	102.0
		1.0	2.974	99.1
		3.0	4.951	98.1

tion procedure and the HPLC conditions are also applicable to plasma samples without interferences due to anticoagulants heparin and/or EDTA. However, sample extracts after derivatization with fluorescamine cannot be loaded into an autosampler because the half-lives of fluorescence intensity of the derivatives are 55–60 min.

Fig. 4 shows the time-course of serum mexiletine concentrations after an intravenous administration to two patients (A,B) who had WPW syndrome. The simplest model for an adequate description of the serum concentrations of mexiletine was a biexponential function ($p < 0.001$, by the distribution F-test). Monoexponential analysis did not significantly increase the goodness of fit ($p > 0.05$). The distribution phase in both patients was observed within 3 h, and after 3 h the serum concentrations were 0.40 and 0.25 μg/ml, respectively. The elimination half-lives ($t_{1/2\beta}$) were 18.2 and 17.7 h, the areas under the curve ($AUC^{0-\infty}$) were 12.5 and 7.60 μg h/ml, and the apparent volumes of distribution (V_d) were 5.3 and 6.8 l/kg, respectively.

There have been several reports of the pharmacokinetics after intravenous administration of mexiletine. Ohashi *et al.* [26] found a $t_{1/2\beta}$ of 10.2 ± 1.2 h, a V_d of 2.1 ± 0.49 l/kg and an $AUC^{0-\infty}$ of 8.35 ± 0.79 μg h/ml. Brockmeyer *et al.* [27] found a $t_{1/2\beta}$ of 15.6 ± 3.7 h, a V_d of 608 ± 193 l and an $AUC^{0-\infty}$ of 12.8 ± 7.88 μg h/ml. The pharmacokinetic parameters estimated in the present study are in general agreement with the results described by these investigators.

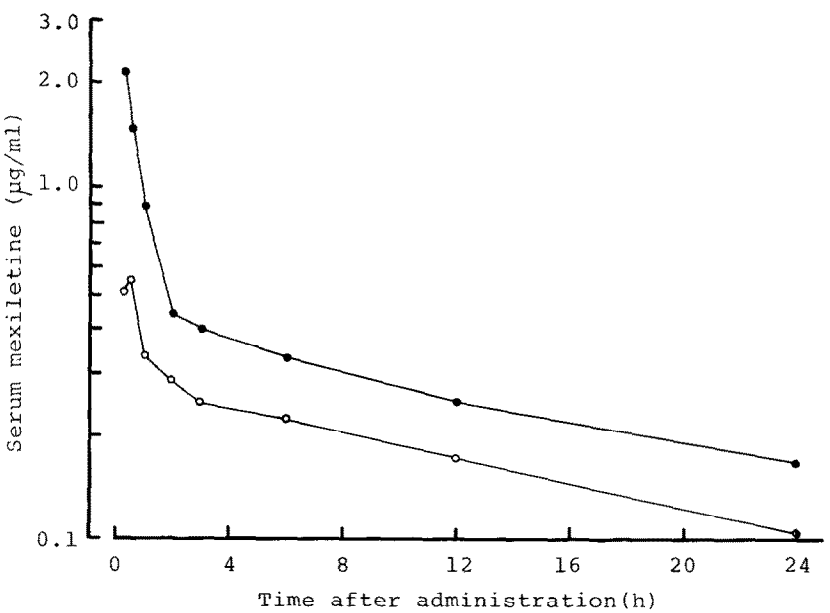

Fig. 4. Serum concentration–time curves of mexiletine in two patients after intravenous administration: (●) patient A, 56 years, 2.5 mg/kg intravenous dose; (○) patient B, 7 years, 2.0 mg/kg intravenous dose.

In conclusion, the present HPLC method with pre-column fluorimetric derivatization by fluorescamine provides a highly sensitive and reliable assay procedure for serum analysis of mexiletine. The serum volumes required are small, the extraction is rapid and simple, and the fluorimetric detection system has the reliability and sensitivity to facilitate measurement of serum levels of mexiletine following oral or intravenous administration of this potent antiarrhythmic drug.

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