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LIQUID CHROMATOGRAPHIC DETERMINATION OF MEXILETINE AND TOCAINIDE IN HUMAN PLASMA WITH FLUORESCENCE DETECTION AFTER REACTION WITH A MODIFIED *o*-PHTHALALDEHYDE REAGENT

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SUMMARY

A liquid chromatographic procedure for the determination of mexiletine or tocainide in human plasma is described. Plasma, after the addition of a homologue of mexiletine or of tocainide, is extracted with dichloromethane. The extract is evaporated and reconstituted in a non-aqueous *o*-phthalaldehyde—mercaptoethanol reagent. An aliquot of the solution is chromatographed on a reversed-phase Ultrasphere-octyl column. The peaks are detected by fluorescence (λ_{ex} = 350 nm and λ_{em} = 445 nm). The fluorescent derivatives of the drugs and internal standards are stable at room temperature and give symmetrical single peaks. Use of fluorescamine as a reagent to prepare fluorescent derivatives of mexiletine and tocainide prior to chromatography is also evaluated.

INTRODUCTION

Mexiletine (I) and tocainide (III) (Fig. 1) are analogues of lidocaine which have been shown to have antiarrhythmic effect in man. They are being evaluated as alternative drugs to lidocaine as they can be administered orally and have relatively long half-lives [1—4]. However, these drugs have a low therapeutic index, and monitoring their therapeutic concentration is considered useful. The effective concentration of mexiletine is considered between 0.2 and 1 mg/l [1, 5] and of tocainide between 6 and 12 mg/l [4].

Gas chromatographic (GC) procedures with electron-capture detection after the preparation of fluoroacyl derivatives of mexiletine [6] and of tocainide [7—10] have been described. Mexiletine has also been determined with flame-ionization detection without derivatization [11] or after the preparation of an

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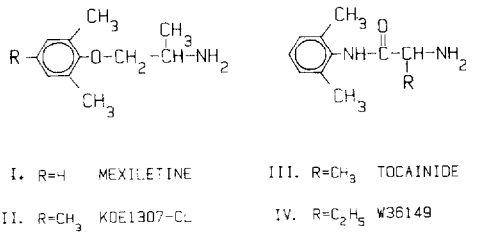

Fig. 1. Structural formulae of compound I—IV.

acetyl derivative [12]. Both mexiletine [13—15] and tocainide [16] have been determined by GC with the use of a nitrogen-selective detector. In another approach tocainide has been determined by GC with nitrogen-selective detection after Schiff base formation [17].

In the last few years there has been an increasing trend to monitor cardiac drugs by liquid chromatography (LC). The simplest approach for the determination of these drugs after LC separation has been to monitor absorbance of mexiletine at 254 nm [18] or at 210 nm [19] and of tocainide at 230 nm [20] or at 210 nm [21]. Mexiletine has also been determined by monitoring its native fluorescence under non-specific conditions [22] after LC separation. In a number of procedures both mexiletine and tocainide have been derivatized prior to LC separation for improved sensitivity and specificity of detection. Thus mexiletine has been determined by monitoring its absorbance at 352 nm after the preparation of its 2,4-dinitrophenyl derivative [23]. Mexiletine [24] and tocainide [25] have been determined by monitoring fluorescence after the preparation of their Dns derivatives prior to LC. Tocainide has also been determined by monitoring fluorescence after pre-column derivatization with fluorescamine [26]. This approach has recently been applied for the determination of mexiletine [27].

In the last few years, compounds containing primary amino groups have been determined with high sensitivity and selective detection after reaction with *o*-phthalaldehyde (OPA) reagent [28—33]. We describe conditions for the determination of mexiletine and tocainide by LC with fluorescence detection after treatment with OPA reagent.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Solvents had been distilled in glass by the supplier (Caledon Labs., Georgetown, Canada). Deionized water was distilled in an all-glass still.

OPA reagent

A 1 g/l solution of *o*-phthalaldehyde (Sigma) was prepared by dissolving 20 mg of *o*-phthalaldehyde in methanol (A). Mercaptoethanol solution was prepared every week by diluting 10 μl of 2-mercaptoethanol (Sigma) in 10 ml of methanol and stored at 4°C (B). Working OPA reagent was prepared when required by mixing 1.5 ml of A and 0.5 ml of B.

Standards

Stock solutions of mexiletine (2 g/l) and tocainide (5 g/l) were prepared by dissolving appropriate amounts of their hydrochlorides in methanol. These solutions were stable for at least six months when stored at 4°C. Plasma-matrix drug standards of mexiletine (10 mg/l) and tocainide (25 mg/l) were prepared by diluting 0.5 ml of methanolic stock solutions to 100 ml with drug-free pooled plasma. Additional plasma standards were prepared by serial dilutions. These standards were stored at −15°C in 1-ml portions. The stock internal standard solutions were prepared by dissolving 20 mg of II or of IV (Fig. 1) in 10 ml of methanol. Working internal standard solution for mexiletine was prepared by diluting 50 μl of II with 100 ml of 1 *M* sodium bicarbonate and for tocainide 100 μl of stock IV with 10 ml of 1 *M* sodium bicarbonate.

Specimen collection

Blood for mexiletine or tocainide assay was collected in green-capped heparinized vacutainer tubes (Becton Dickinson, Orangeburg, NY, U.S.A.). The tubes were centrifuged within 2 h of blood collection and plasma was collected with Pasteur pipettes and stored in disposable plastic tubes at −15°C until analyzed.

Sample preparation

To 0.5 ml of plasma in PTFE-lined screw-capped culture tubes (16 × 100 mm), 0.5 ml of working internal standard and 6 ml of dichloromethane were added. The contents of the tubes were mixed by rotating the tubes on a rotary mixer for 10 min. The tubes were centrifuged and the upper aqueous layer was discarded. To each tube 2 g of anhydrous sodium sulphate were added. The tubes were vortex-mixed and centrifuged. The dichloromethane layer was transferred to correspondingly labelled 16 × 100 mm disposable glass tubes. The extract was evaporated at 45—50°C. The residue in each tube was dissolved in 100 μl of OPA reagent and 5 μl were injected into the chromatograph after 0.5 h.

Chromatography

The chromatographic separation was performed isocratically at room temperature with a single-piston reciprocating pump (Model 110A, Beckman Instruments). Injections were made with a syringe loading injector with a 20-μl loop (Model 7125, Rheodyne). The peaks were detected with a fluorescence detector (Model RF-530, Shimadzu) at λ_{ex} = 350 nm and λ_{em} = 445 nm. A 15 cm × 4.6 mm Ultrasphere-octyl column packed with particles of average diameter 5 μm (Beckman) was used. The column was protected with a guard column (70 × 4.6 mm) packed with Co:Pell ODS of particle size 30—40 μm (Whatman). The mobile phase for mexiletine was prepared by mixing 580 ml of acetonitrile, 420 ml of water, 0.5 ml of 70% perchloric acid and 0.5 ml of 20% methanolic tetramethylammonium hydroxide (TMAH). The mobile phase for tocainide contained 42% acetonitrile rather than 58%. The mobile phase for mexiletine was pumped at 1 ml/min and the mobile phase for tocainide was pumped at 1.5 ml/min. The peaks were recorded with a recording integrator (Model CR 3-A, Shimadzu).

RESULTS AND DISCUSSION

In the last few years the use of OPA reagent both as a pre-column [28—33] and post-column [34] derivatization reagent has gained popularity for sensitive LC determination of compounds containing a primary amino group. Pre-column derivatization is convenient since less equipment is required. However, derivatives prepared prior to chromatography are unstable. The instability of OPA derivatives has been attributed to the presence of water in the reaction mixture. These derivatives have been stabilized either by extraction of the reaction mixture [28] or by dilution of the reaction mixture with methanol [33]. The standard OPA reagent is a dilute solution of *o*-phthalaldehyde and 2-mercaptoethanol in borate buffer of pH ~ 10. The commercially available, ready-to-use OPA reagent sold under the trade name of Fluoraldehyde (Pierce Chemical) is prepared in a specially stabilized borate buffer. In some cases fluorescent products have been prepared by reacting compounds with primary amino group with *o*-phthalaldehyde without the presence of thiols [35]. However, the reaction conditions and the nature of the product formed are different from the commonly used OPA reagent containing thiols. Thiols other than 2-mercaptoethanol have been used for improved stability or enhanced fluorescence [36].

In an attempt to find an OPA reagent without the aqueous buffer, a number of mixtures of *o*-phthalaldehyde and 2-mercaptoethanol solutions were prepared in varying compositions. The pH values of these solutions were varied by adding glacial acetic acid or a methanolic solution of TMAH. These mixtures

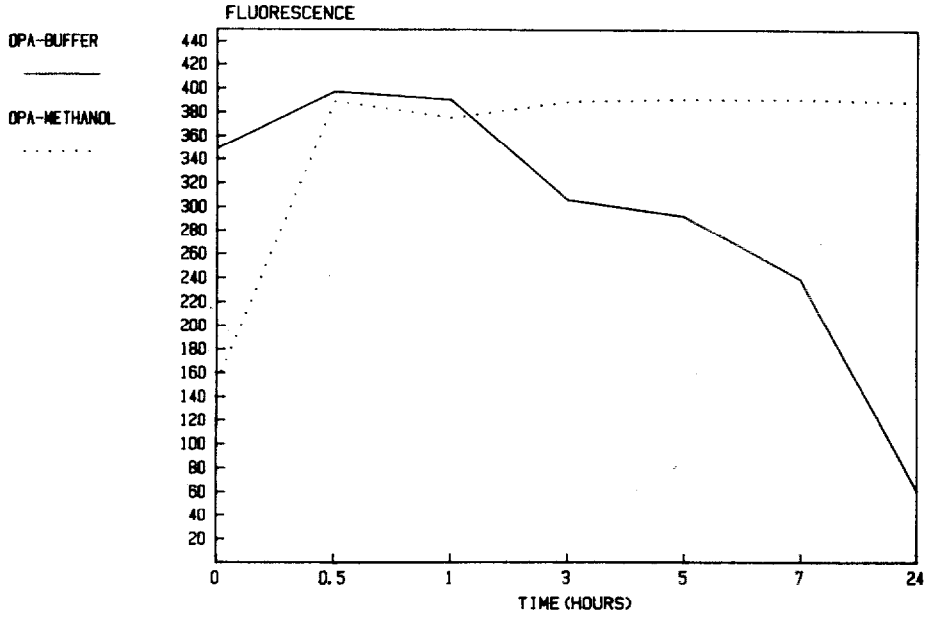

Fig. 2. Comparison of stability of fluorescence of *o*-phthalaldehyde derivatives of mexiletine with and without borate buffer.

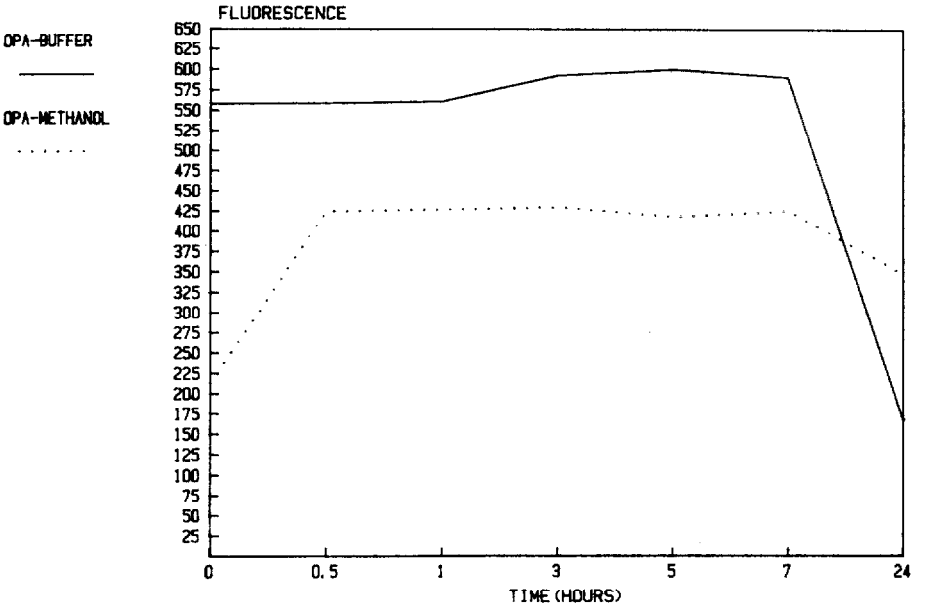

Fig. 3. Comparison of stability of fluorescence of *o*-phthalaldehyde derivatives of tocainide with and without borate buffer.

were evaluated for the presence of reagent peaks, fluorescence response and stability of reaction products with mexiletine and tocainide. The reagent composition described in this report was found most satisfactory in all respects.

We have compared the action of this modified OPA reagent with that of standard OPA reagent with mexiletine (Fig. 2) and tocainide (Fig. 3). The presence of aqueous buffer allows the development of maximal fluorescence relatively rapidly as compared to non-aqueous OPA reagent. However, fluorescence of OPA derivatives of these drugs is relatively less stable in the prescence of aqueous buffer than in the modified reagent. The products formed with mexiletine or tocainide with the two types of reagents show the same retention times when chromatographed using different columns (C_{18}, C_8 or PRP-1) or with mobile phases of different pH with a C_8 column. The fluorescence response of a compound with both types of reagents depends on the structure of the compound. Thus mexiletine gives approximately an order of magnitude higher fluorescence response when compared to that of tocainide with the same OPA reagent.

Fluorescamine as a derivatizing reagent

Fluorescamine, a non-fluorescent compound, is another reagent which, like OPA reagent, reacts with primary amines producing highly fluorescent products [37]. The standard fluorescamine reagent is a mixture of its solution in acetonitrile and an aqueous phosphate or borate buffer of desired pH [37]. We have observed that the fluorescamine derivative of tocainide can be prepared without loss in fluorescence yield but with improved stability in the absence of aqueous buffer (Fig. 4). However, fluorescamine derivatives prepared in the

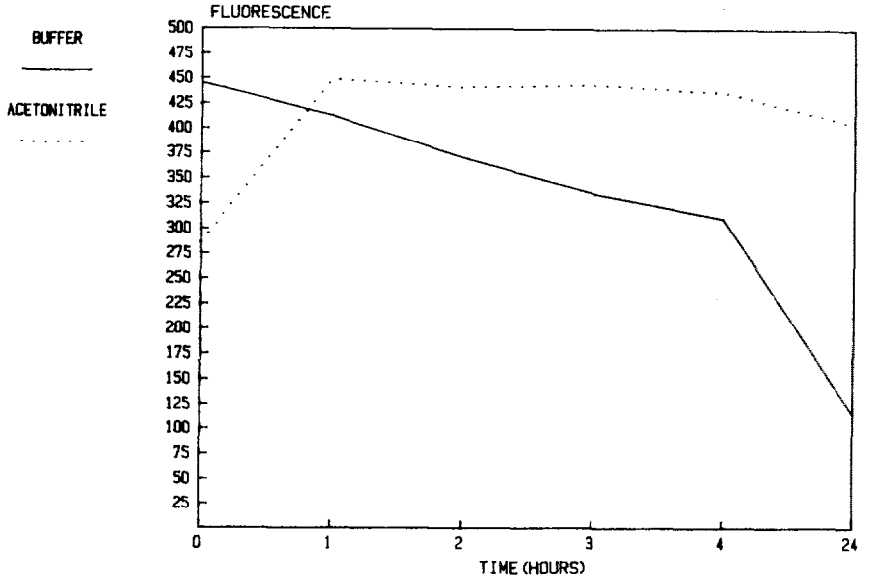

Fig. 4. Comparison of stability of fluorescence of fluorescamine derivatives of tocainide with and without phosphate buffer.

presence of aqueous buffer differ in retention time (0.5 min) from those prepared without aqueous buffer. As is the case with OPA derivatives, fluorescence of fluorescamine derivatives also depends on the structure of the amine. Thus the fluorescamine derivative of tocainide has a much stronger fluorescence than the OPA derivative of tocainide but the fluorescamine derivative of mexiletine has a much weaker fluorescence than the OPA derivative of mexiletine.

Fluorescamine derivatives of mexiletine and tocainide and their corresponding internal standards give double peaks when chromatographed on a C_8 column with acetonitrile—water as the mobile phase. When the pH of the mobile phase is adjusted to approximately 3 with tetramethylammonium perchlorate, the fluorescamine derivative of tocainide gives one peak but the other three compounds still give double peaks. Further change of pH of the mobile phase to approximately 2 did not make any difference. Fluorescamine derivatives of amino acids have been reported to give double peaks when chromatographed on a μBondapak C_{18} column [38]. However, chromatographic conditions have been described when fluorescamine derivatives of tocainide [26], aminocaproic acid [39], clovoxamine [40] and histamine [41] give single peaks.

Assay of mexiletine and tocainide

We selected OPA as a pre-column derivatizing reagent because of its strong fluorescent response with mexiletine which has a relatively low therapeutic range and because of formation of single peaks after derivatization of different compounds with any type of column or pH of the mobile phases. Even the

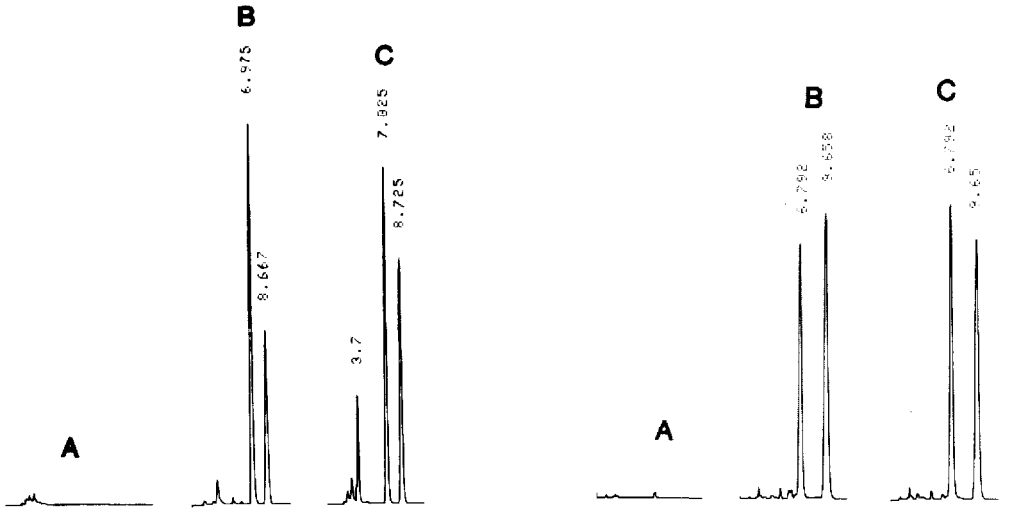

Fig. 5. Chromatograms of drug-free plasma (A), of plasma with added mexiletine (1.0 mg/l) (B) and of plasma of a patient receiving mexiletine (C). Peak of mexiletine corresponds to 0.6 mg/l. Detector sensitivity, high; integrator attenuation, 3; chart speed, 2 mm/min.

Fig. 6. Chromatograms of drug-free plasma (A), of plasma with added tocainide (10 mg/l) (B) and of plasma of patient receiving tocainide (C). Peak of tocainide corresponds to 12.6 mg/l. Detector sensitivity, high; integrator attenuation, 3; chart speed, 2 mm/min.

weak fluorescence response of tocainide allows higher sensitivity with few extraneous peaks than the ultraviolet detection of underivatized tocainide at 230 or 210 nm. We have selected KOE 1307-CL (an analogue of mexiletine) and W36149 (an analogue of tocainide) as internal standards of mexiletine and tocainide, respectively (Fig. 1). These compounds show fluorescence responses similar to the respective drugs after derivatization with OPA and the area ratio of drug to internal standard remains constant with time.

As seen in Figs. 5A and 6A, extraction of plasma with dichloromethane provides clean extracts. The uncorrected analytical recovery of mexiletine and tocainide is 75—80% as determined by analyzing supplemented plasma and comparing the area of the drug peak with that of the peak of the same aliquot of non-extracted standard after treatment with OPA reagent. The recovery was optimal when the extraction was carried out with the use of 1 *M* sodium bicarbonate (pH 8.5) and decreased with buffers of higher or lower pH.

The peaks of drugs and the corresponding internal standards are symmetrical and well resolved (Figs. 5B and 6B). Analysis of plasma obtained from a patient receiving mexiletine (Fig. 5C) shows an additional peak (retention time = 3.7 min). However, analysis of plasma obtained from a patient receiving tocainide (Fig. 6C) shows no additional peak. The ratio of peak areas of mexiletine to internal standard is linearly related to the mexiletine concentration over the range tested (0.1—10 mg/l). Similarly, the peak area ratio of tocainide to internal standard is linear for the range tested (0.5—25 mg/l). There are no peaks from previous injections, and plasma extracts can be injected every 11 min for mexiletine or tocainide. The procedure is satisfactorily reproducible for both of the drugs (Table I).

The presence of commonly prescribed antiarrhythmic drugs which could be co-administered with mexiletine or tocainide was considered. Separation of tocainide from its internal standard can be accomplished fairly rapidly with the mobile phase containing 50% acetonitrile. However, quinidine, which has a strong native fluorescence, would interfere with the assay of tocainide under these conditions. For adequate separation of quinidine, the acetonitrile content of the mobile phase was reduced from 50 to 42% and the flow-rate increased from 1 to 1.5 ml/min. As seen in Fig. 7, the elution order of quinidine and tocainide are reversed with a change in acetonitrile content. Chromatography of mexiletine did not present any special problems. Under the selected

TABLE I

ESTIMATION OF PRECISION (n = 10)

Compound	Concentration (mean ± S.D.) (mg/l)		Coefficient of variation (%)	
	Within batch	Between batch	Within batch	Between batch
Plasma mexiletine	0.25 ± 0.010	0.25 ± 0.014	3.9	5.8
	5.0 ± 0.114	5.0 ± 0.092	2.3	6.1
Plasma tocainide	2.5 ± 0.032	2.5 ± 0.068	1.3	2.7
	14.9 ± 0.38	15.0 ± 0.41	2.8	2.7

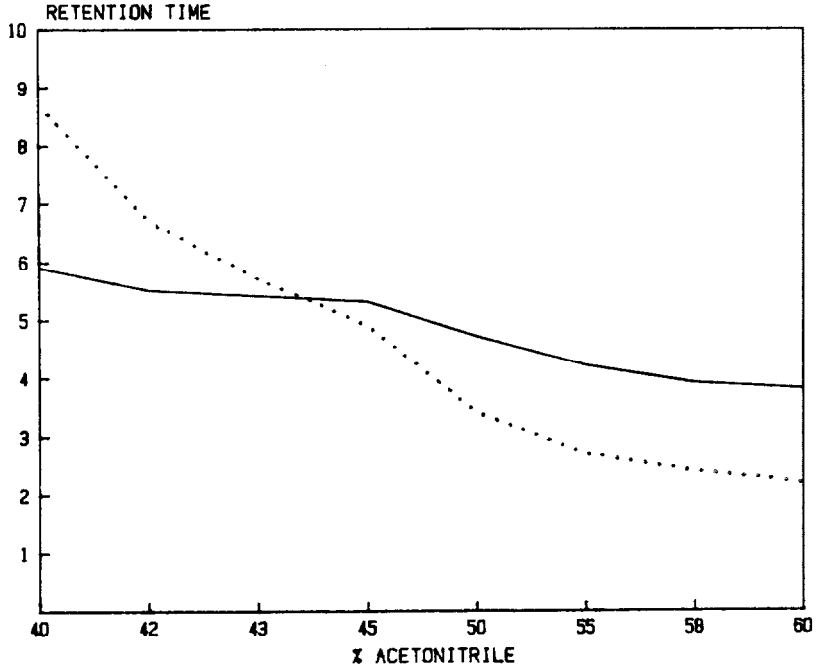

Fig. 7. Effect of acetonitrile content of the mobile phase on the elution of o-phthalaldehyde derivative of tocainide (• • • •) and quinidine (——).

TABLE II

INTERFERENCE STUDIES

Mobile phase A: acetonitrile—water—20% tetramethylammonium hydroxide—70% perchloric acid (580:420:0.5:0.5); flow-rate = 1.0 ml/min. Mobile phase B: acetonitrile—water—20% tetramethylammonium hydroxide—70% perchloric acid (420:580:0.5:0.5); flow-rate = 1.5 ml/min.

Drug	Concentration (mg/l)	Retention time (min)	
		Mobile phase A	Mobile phase B
N-Acetylprocainamide	10	—*	—
Amiodarone	20	—	—
Amphetamine	10	— (6.0)**	—
Diazepam	1	—	—
Disopyramide	20	—	—
Lidocaine	20	—	—
Mexiletine	1	7.0	—
KOE 1307-CL	0.5	8.7	—
Phenylpropanolamine	10	3.2	5.2
Procainamide	40	—	—
Propranolol	1	—	—
Quinidine	10	5.6	5.5
Tocainide	5	3.4	6.7
W36149	10	4.0	9.6
Trazodone	4	4.3	5.9

*Dash signifies no peak between 2 and 20 min.
**Lost during evaporation, retention time of OPA derivative of unextracted amphetamine.

conditions no interference has been observed when plasma supplemented with a number of commonly prescribed antiarrhythmic drugs and other compounds was analyzed by the present procedure (Table II). Similar separation can also be achieved with use of a C_{18} column. However, a higher concentration of acetonitrile is required.

In conclusion, the described procedure is an acceptable alternative LC procedure for the determination of mexiletine and tocainide in plasma. There is an increase in sensitivity and specificity of detection by merely dissolving the plasma extract in a reagent rather than in the mobile phases as compared to UV detection at 210 nm.

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