Journal of Chromatography, 415 (1987) 335–346
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3528

STEREOSELECTIVE ANALYSIS OF THE ENANTIOMERS OF MEXILETINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTION AND STUDY OF THEIR STEREOSELECTIVE DISPOSITION IN MAN

K.M. McERLANE* and L. IGWEMEZIE

Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia (Canada)

and

C.R. KERR

Faculty of Medicine, University of British Columbia, Vancouver, British Columbia (Canada)

(First received August 28th, 1986; revised manuscript received December 1st, 1986)

SUMMARY

A sensitive, stereoselective high-performance liquid chromatographic assay was developed for the resolution of the enantiomers of mexiletine as their 2-naphthoyl derivatives on a Pirkle® type 1A chiral phase column. Detection of the derivatives was accomplished with a fluorescent detector. Maximum recovery of the enantiomers from plasma was 83% and was observed when plasma proteins were precipitated with a mixture of barium hydroxide–zinc sulphate. The calibration curve in plasma was linear over the concentration range 5–750 ng/ml for each enantiomer ($r^2 = 0.999$) and in urine the linear range was 0.25–7.5 μg/ml ($r^2 = 0.999$) for each enantiomer. The minimum detectable quantity of each enantiomer in plasma was 5 ng/ml at a signal-to-noise ratio of 5:1, representing 100 pg injected. A preliminary pharmacokinetic study was undertaken in one healthy male volunteer following an oral dose of 300 mg of racemic mexiletine hydrochloride. The apparent elimination half-lives determined from the plasma data were 12.1 and 14.1 h for the $R(-)$ and $S(+)$ enantiomers, respectively. The cumulative urinary excretion amounts of $R(-)$- and $S(+)$-mexiletine were found to be 8.01 and 10.46 mg, respectively. The plasma data indicated that a cross-over of the enantiomer ratios occurred at approximately 8 h. The urinary excretion of the enantiomers was consistent with the pattern found in plasma.

INTRODUCTION

Mexiletine, [1-(2′, 6′-dimethylphenoxy)-2-propanamine], is a relatively new class 1 antiarrhythmic agent with similar structural and electrophysiological effects to that of lidocaine [1]. Unlike lidocaine, however, mexiletine is effective

0378-4347/87/$03.50

by the oral route and exhibits a longer elimination half-life. It is therefore useful in the treatment of acute and long-term ventricular arrhythmias [2]. Mexiletine has a chiral centre in its molecular arrangement and is used therapeutically as the racemic mixture. A number of drugs in use today contain chiral centres and it has been established that the enantiomers of many of these agents exhibit differing pharmacokinetic and/or pharmacological activities. Recent reports by Simonyi [3] and Williams and Lee [4] reviewed the enantioselective properties of many of these chiral drugs. At the present time there is no information on the activity of the individual enantiomers of mexiletine; however, there have been a number of reports on the analysis and pharmacokinetics of the racemic form of mexiletine [5–11] and one previous manuscript on the analysis and preliminary pharmacokinetics of the individual enantiomers in humans [12]. The achiral methods include gas chromatographic (GC) procedures [5–8] and high-performance liquid chromatographic (HPLC) procedures [9–11]. However, with recent advances in stereoselective assay methodology, it would seem appropriate to also examine the individual enantiomers in human subjects.

The previously reported [12] stereoselective HPLC method required the preparation of diastereoisomers of the enantiomers using the chiral reagent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC). While resolution of the enantiomers was achieved, there are drawbacks in such procedures such as: the need for enantiomerically pure chiral reagents and the quantitation errors that may occur as a result of the differing rates of reaction of the enantiomers [13]. These disadvantages are not inherent in resolution of enantiomers by the use of chiral chromatographic stationary phases. Accordingly, the enantiomers of mexiletine were resolved on a chiral column (Pirkle Phase®). The use of a naphthoyl derivative facilitated sensitive fluorescent detection and increased the sensitivity of measurement considerably over that reported earlier.

EXPERIMENTAL

Materials

R,S-Mexiletine hydrochloride, *R,S*-mexiletine hydrochloride (Mexitil®) capsules (100 and 200 mg), *R*(−)- and *S*(+)-mexiletine hydrochloride and the internal standard, 1-(2′,6′-dimethylphenoxy)-2-ethanamine, were kindly supplied by Boehringer Ingelheim (Burlington, Canada). 2-Naphthoyl chloride was obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC grade *n*-hexane, diethyl ether and 2-propanol were obtained from BDH Chemicals (Vancouver, Canada) as were barium hydroxide octahydrate and zinc sulphate heptahydrate. All other chemicals were of analytical grade and were used without further purification.

Equipment

HPLC. A Hewlett-Packard liquid chromatograph Model 1082B, equipped with a Model 79850B data terminal (Hewlett-Packard, Avondale, PA, U.S.A.), a 20-μl fixed volume loop injector (Rheodyne, Berkeley, CA, U.S.A.) and a Model 970 Fluorometer (Kratos, Westwood, NJ, U.S.A.) were used throughout. The excitation wavelength of the fluorometer was set at 230 nm and a 340-nm cut-off

filter was used for the emission wavelength. The HPLC column was a 25×0.46 cm Pirkle type 1A column consisting of 3,5-dinitrobenzoyl-phenylglycine, ionically bonded to a 5-μm silica gel support (Regis, Morton Grove, IL, U.S.A.). The mobile phase consisted of 2-propanol—*n*-hexane (5.5:94.5) and was delivered isocratically at 1.4 ml/min.

Gas chromatography–mass spectrometry (GC–MS). A Hewlett-Packard Model 5987A GC–MS system equipped with a Model 5880 gas chromatograph (Hewlett-Packard) was operated in the positive chemical ionization mode for determination of the structure of the naphthoyl derivatives of mexiletine and the internal standard. The GC column was a fused-silica capillary column (12 m×0.2 mm I.D.) with cross-linked methyl silicone as the stationary phase (Hewlett-Packard). The following splitless GC conditions were used: helium carrier gas flow-rate, 1 ml/min; injection port temperature, 240 °C; oven temperature programme was ramped from 50 °C for 1 min to 300 °C for 10 min at 30 °C/min. The following MS conditions were used: interface temperature, 240 °C; ion source temperature, 240 °C; electron multiplier voltage, 2500 V; emission current, 300 μA and methane was used as the reagent gas.

Stock solutions

R,S-Mexiletine stock solution was prepared by dissolving 1.2039 mg of *R,S*-mexiletine hydrochloride (equivalent to 10 μg/ml of the base) in 100.0 ml of deionized, distilled water. Dilution of 10 ml of this solution to 100.0 ml with distilled water gave a lower concentration stock solution of 1 μg/ml equivalent of the base. A solution of the internal standard was similarly prepared by dissolving 0.4976 mg of the hydrochloride salt in 100.0 ml of deionized, distilled water.

Barium hydroxide (0.15 *M*) solution was prepared by dissolving 4.7 g of barium hydroxide octahydrate in 100 ml of deionized, distilled water and 5% zinc sulphate heptahydrate was prepared by dissolving 5.0 g of zinc sulphate heptahydrate in 100 ml of distilled water.

The derivatization reagent, 2-naphthoyl chloride, was prepared by dissolving 2 mg in 2 ml of dichloromethane.

Calibration curve and assay precision

Plasma. To six 1-ml aliquots of plasma were added 10, 100, 200, 500, 1000 and 1500 ng (equivalent of the base) of *R,S*-mexiletine from the stock solutions along with 0.05 ml of the internal standard working solution (equivalent to 200 ng of the base). The plasma proteins were precipitated with 1 ml of barium hydroxide solution and 1 ml of zinc sulphate solution according to the method of Somogyi [14]. The plasma pH was adjusted above 12 by the addition of 0.4 ml of 2 *M* sodium hydroxide and extracted twice with 5-ml portions of diethyl ether. The organic extracts were combined and evaporated at 37 °C under a gentle stream of clean, dry nitrogen to a volume of approximately 1 ml. The resulting solution was acidified with 0.2 ml of 0.1 *M* hydrochloric acid, shaken, and the ether layer was removed and discarded. The aqueous layer was again basified with 0.2 ml of 2 *M* sodium hydroxide and 15 μl of the 2-naphthoyl chloride solution were added and mixed vigorously on a vortex mixer (Thermolyne, Sybron, Dubuque, IA, U.S.A.)

for 2 min. The derivatives formed were extracted into 0.6 ml of the mobile phase used for the HPLC analysis [2-propanol–hexane (5.5:94.5)]. For the 10-ng sample, the extract was evaporated to dryness and reconstituted with 120 μl of the mobile phase. A 20-μl aliquot was injected onto the HPLC column. The calibration curves were constructed by plotting the peak height ratios of each enantiomer to that of the internal standard against the known concentrations of the enantiomers. Inter-assay variability was determined by triplicate preparation and analysis of each of the samples used for the standard curve. Intra-assay variability was determined by triplicate injections of three of the solutions (containing 100, 500 and 1500 ng) used for determination of the calibration curve linearity.

Urine. To six 0.2-ml aliquots of urine were added 100, 500, 1000, 1500, 2000 and 3000 ng (equivalent of the base) of the *R,S*-mexiletine working solution along with 0.075 ml of the internal standard working solution (equivalent to 300 ng of the base). The solutions were basified by the addition of 0.4 ml of 2 *M* sodium hydroxide solution and were extracted with two 5-ml aliquots of diethyl ether. The ethereal extract was then treated in the same manner as described for the plasma extracts.

Recovery of mexiletine from plasma

The recovery of mexiletine enantiomers from plasma was determined by the addition of 100, 200, 500, 1000 and 1500 ng of *R,S*-mexiletine to five 1-ml aliquots of plasma obtained from a volunteer. The samples were extracted, derivatized and assayed as described above except that the internal standard was added just prior to the derivatization procedure. The resulting peak height ratios were expressed as a percentage of those obtained with identical amounts of *R,S*-mexiletine and internal standard prepared in water but which were derivatized directly without extraction into diethyl ether.

In vivo study

A healthy male volunteer (100 kg) who had fasted overnight was given an oral dose of 300 mg of mexiletine hydrochloride in capsule form (one 100-mg plus one 200-mg capsule) with approximately 100 ml of water. Venous blood samples were collected into heparinized evacuated tubes (Becton Dickinson, Rutherford, NJ, U.S.A.) via an indwelling 19 gauge Butterfly cannula (Abbott Labs., Montreal, Canada) inserted in a vein on the back of the forearm. Samples (8 ml) were withdrawn at 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 6, 8, 10, 12, 14, 16, 25, 30, 36 and 48 h. The cannula was flushed with 1 ml of a sterile, isotonic solution of heparin in sodium chloride (50 international units/ml) after each withdrawal and the heparin was cleared into an evacuated tube prior to each blood sampling. Blood samples were centrifuged at 2500 *g* for 15 min and the plasma was separated and stored at −20 °C until required for analysis. Urine samples were collected in plastic Whirl Pak® bags (Canlab, Vancouver, Canada) at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 25, 30, 36 and 48 h, and at the convenience of the subject after the 16-h sample. The urine pH was measured immediately after collection and the volumes were recorded before the samples were frozen at −20 °C.

Assay of plasma and urine samples

To 1-ml aliquots of plasma was added 0.05 ml of the internal standard working solution and the plasma proteins were precipitated with 1 ml each of the barium hydroxide and zinc sulphate solutions. The samples were basified, extracted and derivatized as described under *Calibration curve and assay precision.* For urine samples, 0.2-ml aliquots were taken and 0.075 ml of the internal standard working solution was added to each. The urine samples were basified, extracted and derivatized as described under *Calibration curve and assay precision.* In order to remove endogenous material from the column it was necessary to wash the column with 2-propanol—hexane (10:90) for 10 min after every second injection of the urine extracts.

RESULTS AND DISCUSSION

The initial attempts at the resolution of the enantiomers of mexiletine was studied using a chiral HPLC stationary phase consisting of a covalently bonded 3,5-dinitrobenzoyl-phenylglycine phase (Pirkle covalent chiral column) and a mobile phase of varying proportions of 2-propanol in hexane. Due to the nature of the Pirkle column, primary amines exhibit long retention times and therefore this group is normally reacted with a reagent to reduce the basicity. Accordingly, derivatization of the mexiletine enantiomers with trifluoroacetic anhydride provided partial resolution ($R=0.45$) which could not be improved by alterations in the composition of the mobile phase. However, pentafluoropropionyl and heptafluorobutyryl derivatives did not provide any resolution. It was considered that the fluorine atoms of the latter halogenated derivatives were responsible for a decrease in interaction with the chiral stationary phase due to their electronegativity and/or their greater bulk. The non-halogenated acetamide, on the other hand, provided significantly greater resolution ($R=1.45$). While resolution of this derivative was near baseline, the sensitivity of detection by UV was insufficient for pharmacokinetic studies. Accordingly, the 2-naphthoyl derivatives were formed from the two enantiomers and these not only provided good resolution ($R=1.45$) but allowed for the use of fluorescent detection of as little as 5 ng of each isomer isolated from plasma at a signal-to-noise ratio of 5:1. Later studies for pharmacokinetic measurements were conducted on the Pirkle type 1A ionic column which provided slightly better efficiency and resolution.

The rate of reaction of mexiletine enantiomers and the internal standard using the normal conditions of the Schotten–Baumann reaction [15] was determined by preparation of six identical samples and shaking these for 1, 5, 10, 15, 20 and 30 min at room temperature. Analysis of these samples using absolute peak height comparisons showed no detectable differences between the resulting peaks. Therefore, the reaction appears to be complete within 1 min or less and the derivatives were found to be stable for at least 14 days. The structural identity of the 2-naphthoyl derivatives were determined by GC–MS. The resulting mass spectra of the derivatives of *R,S*-mexiletine and the internal standard are shown in Fig. 1 and the proposed fragmentation pathways in Fig. 2. The total ion current chromatogram did not contain any evidence of other substances.

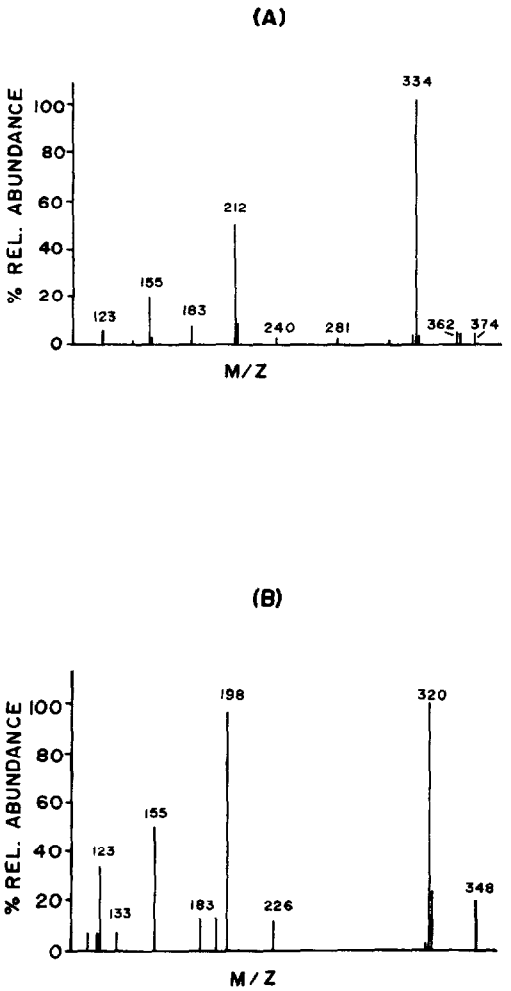


Fig. 1. Positive chemical ionization mass spectrum of mexiletine-2-naphthamide derivative (A) and the internal standard as its naphthamide derivative (B).

Resolution of enantiomers on the Pirkle chiral stationary phase has been reported for a number of primary amines, amino alcohols and amino acids [16,17]. The mechanism of this resolution has been proposed to be due to the transient formation of diastereomeric complexes between the enantiomers and the chiral stationary phase. The relative stability of these complexes results in differing rates of elution of the enantiomers. The complexes are formed as a result of π-bonding, electrostatic bonding, hydrogen bonding and a steric interaction. Hence the enantiomers of mexiletine are envisaged to interact due to π-bonding between the π-basic naphthoyl group of the mexiletine derivatives and the π-acidic 3,5-dinitrobenzoyl group of the chiral stationary phase. Electrostatic bonding of the amide dipoles and a steric interaction of the methyl and xylyloxymethyl portions of the chiral centre of mexiletine with the proximate portion of the stationary

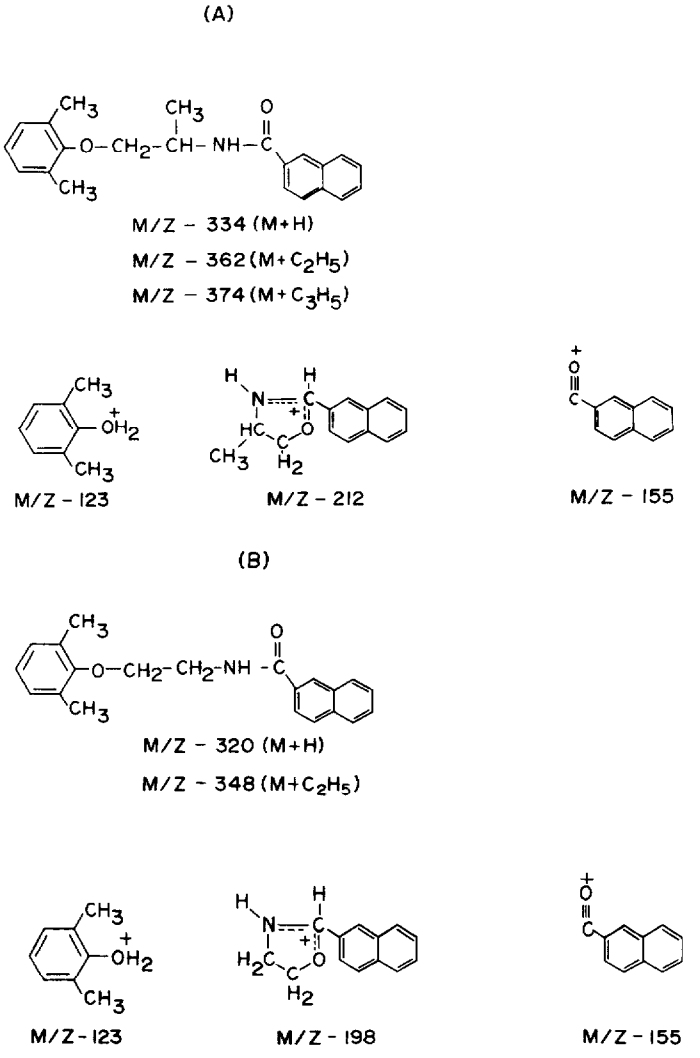
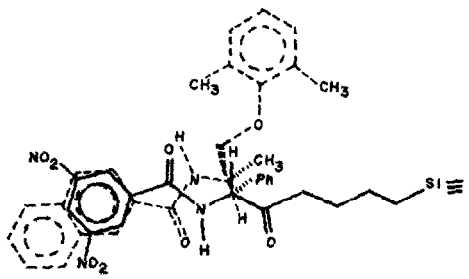


Fig. 2. Proposed fragment ions of mexiletine-2-naphthamide derivative (A) and the internal standard as its naphthamide derivative (B).

phase also contribute to the closeness of fit of the two mexiletine enantiomers. As shown in Fig. 3, a more stable complex is formed with $S(+)$-mexiletine-2-naphthamide where the methyl group lies below the plane of the analyte molecule. The antipode will have the bulkier xylyloxymethyl portion below the plane of the molecule and hence will be more sterically hindered from forming a closer fit with the chiral stationary phase. These interactions lead to a more rapid elution of the $R(-)$ enantiomer. Further confirmation of the elution order was provided by analysis of the individual enantiomer derivatives of mexiletine on the chiral stationary phase.

For the purpose of determining the applicability of the stereoselective method



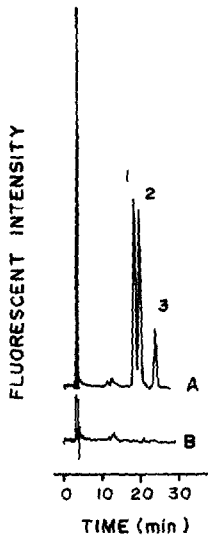


Fig. 3. Proposed stereochemical interaction between $S(+)$-mexiletine-2-naphthamide (dotted structure) and the Pirkle chiral stationary phase (solid structure).

Fig. 4. Chromatogram of $R(-)$-mexiletine-2-naphthamide (1), $S(+)$-mexiletine-2-naphthamide (2) and the internal standard as its naphthamide derivative (3), isolated from plasma (trace A). Sample contained 1 μg/ml of racemic mexiletine and 200 ng/ml of the internal standard. Detection was by fluorescence: excitation, 230 nm; emission, 340 nm cut-off filter. Trace B was obtained from the plasma blank.

in plasma and urine samples, racemic mexiletine was added to blank plasma samples obtained from a variety of donors. As shown in Fig. 4, the resulting chromatograms obtained from a spiked plasma sample and its respective blank displayed no evidence of interference from endogenous substances with the peaks due to the drug enantiomers and the internal standard. In a similar fashion, urine samples prepared and assayed did not show any evidence of interference.

A study of the reovery of the enantiomers of mexiletine from plasma showed substantially low recovery as well as an alteration in the natural enantiomeric ratio of the racemate. Such differences were dependent on the method used to precipitate plasma proteins. When 2 *M* sodium hydroxide was employed to precipitate proteins and to basify the plasma for extraction, the recovery of the enantiomers of mexiletine was unacceptably low and the quantity of the $R(-)$ enantiomer was lower than its antipode. Protein precipitation with 10% trichloroacetic acid enhanced recoveries and restored the natural racemic ratio; however, it was found to produce large variations in the precision of recovery. The procedure reported by Somogyi [14] which employed precipitation with a mixture of barium hydroxide and zinc sulphate solutions, was found to lead to reliably good recoveries ($82.7 \pm 1.3\%$, $n=5$). Representative chromatograms are shown in Fig. 5. Of particular note in these chromatograms is the relationship of the ratio and recovery of the enantiomers extracted from water and those extracted from plasma in which the proteins were precipitated with sodium hydroxide and

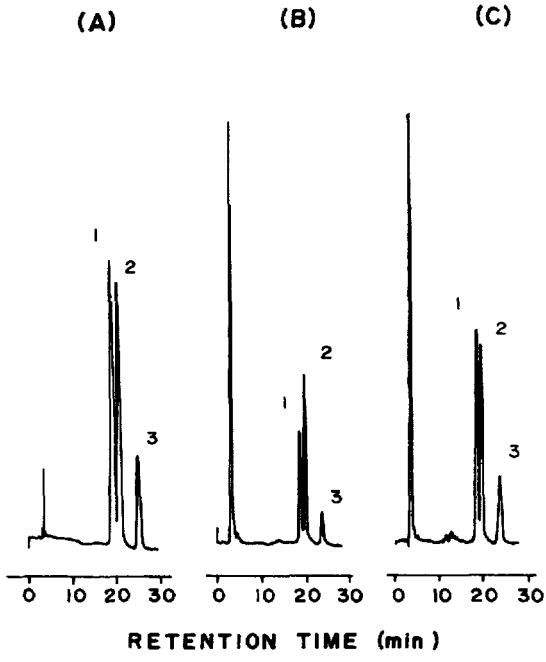


Fig. 5. Chromatograms showing the relationship of mexiletine enantiomers extracted from water (A), from plasma in which proteins were precipitated with sodium hydroxide (B), and plasma in which proteins were precipitated with zinc sulphate—barium hydroxide (C). Refer to Fig. 4 for peak identities, quantities of drug and internal standard and detection conditions.

plasma in which the proteins were precipitated with barium hydroxide—zinc sulphate solution. The natural enantiomeric ratio was maintained with the latter method of protein precipitation and is identical to that seen in water.

The calibration curve data and precision of assay in both plasma and urine are

TABLE I

CALIBRATION CURVE DATA FOR MEXILETINE ENANTIOMERS IN PLASMA

Weight of each enantiomer (ng)	Peak-height ratio $R(-)$-mexiletine/internal standard*	Peak-height ratio $S(+)$-mexiletine/internal standard*
5	0.028 ± 0.001	0.027 ± 0.001
50	0.334 ± 0.011	0.314 ± 0.012
100	0.630 ± 0.017	0.593 ± 0.013
250	1.571 ± 0.028	1.481 ± 0.033
500	3.003 ± 0.079	2.821 ± 0.073
750	4.565 ± 0.027	4.270 ± 0.054
Slope	0.0060	0.0056
Intercept	0.0226	0.0250
r^2	0.9998	0.9997

*Mean ± S.D.; $n = 3$.

TABLE II

CALIBRATION CURVE DATA FOR MEXILETINE ENANTIOMERS IN URINE

Weight of each enantiomer (ng)	Peak-height ratio $R(-)$-mexiletine/internal standard*	Peak-height ratio $S(+)$-mexiletine/internal standard*
50	0.205 ± 0.003	0.187 ± 0.002
250	0.982 ± 0.002	0.921 ± 0.006
500	2.000 ± 0.078	1.879 ± 0.071
750	2.974 ± 0.037	2.796 ± 0.046
1000	3.862 ± 0.062	3.600 ± 0.035
1500	5.831 ± 0.044	5.499 ± 0.026
Slope	0.0039	0.0036
Intercept	0.0304	0.0201
r^2	0.9998	0.9996

*Mean ± S.D.; $n = 3$.

given in Tables I–III. Peak height measurements were found to provide linear calibration curve results ($r^2 = 0.999$) over the concentration range 5–750 ng/ml for each enantiomer in plasma and a similar correlation was found in urine over the concentration range 0.25–7.5 μg/ml for each enantiomer. The intra- and inter-assay coefficients of variation were less than 4% for both biological fluids.

The applicability of the assay was shown in a preliminary pharmacokinetic study conducted on one individual. An oral dose of 300 mg of mexiletine hydrochloride was given to a fasting healthy male (100 kg, 188 cm) and blood and urine samples were collected as outlined in the Experimental section. The plasma concentration–time curve shown in Fig. 6, fitted by a non-linear least squares computer program, Autoan [18] indicated a two-compartment open-model with rapid absorption of both enantiomers within 2.5 h. The terminal elimination half-lives, using equal weighting of all data points, were 12.1 and 14.1 h for the $R(-)$ and $S(+)$ enantiomers, respectively. It was also noted that the ratio of the two enantiomers reversed at approximately 8 h. A similar reversal was noted in the urinary excretion of the enantiomers (Fig. 7) at 4.5 h. The area under the plasma con-

TABLE III

INTRA-ASSAY VARIABILITY OF MEXILETINE ENANTIOMERS IN PLASMA

Weight of each enantiomer (ng)	Peak-height ratio $R(-)$-mexiletine/internal standard*	C.V.** (%)	Peak-height ratio $S(+)$-mexiletine/internal standard*	C.V ** (%)
50	0.324 ± 0.005	1.5	0.302 ± 0.006	1.9
250	1.582 ± 0.013	0.8	1.491 ± 0.022	1.5
750	4.549 ± 0.058	1.3	4.266 ± 0.078	1.9

*Mean ± S.D. ($n = 3$) of one sample at each concentration.
**Coefficient of variation.

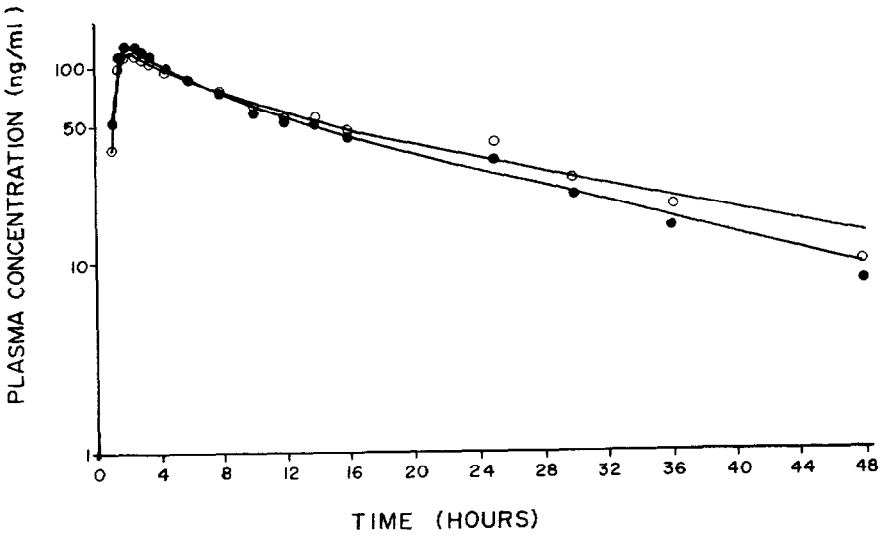


Fig. 6. Plasma concentration—time curve for mexiletine enantiomers. ○, $S(+)$ enantiomer; ●, $R(-)$ enantiomer.

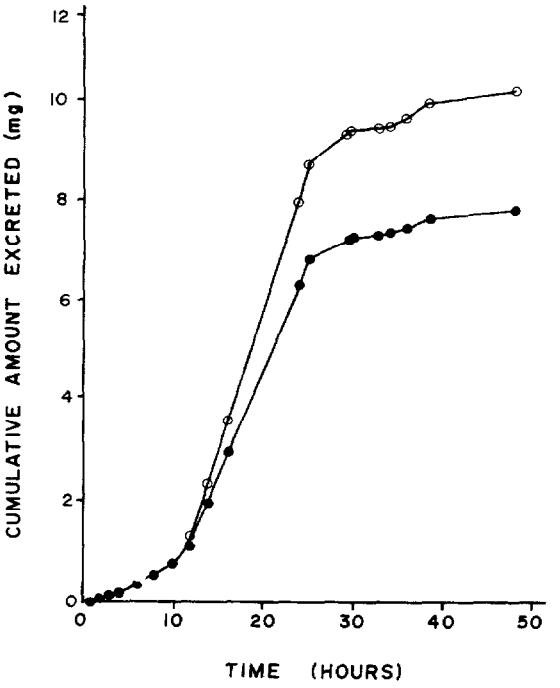


Fig. 7. Cumulative urinary excretion of mexiletine enantiomers. ○, $S(+)$ enantiomer; ●, $R(-)$ enantiomer.

centration–time curve was calculated by the trapezoidal rule and was found to be 2.04 μg/ml·h and 2.24 μg/ml·h for the $R(-)$ and $S(+)$ enantiomers, respectively. The cumulative urinary excretion of $R(-)$-mexiletine was 8.01 mg and that of the $S(+)$-enantiomer was 10.46 mg and are consistent with the plasma data.

In summary, the stereoselective HPLC procedure developed allows for a relatively simple and sensitive analysis of mexiletine enantiomers and can provide quantitative data for up to 48 h in plasma and up to 72 h in urine. The method therefore allows for a longer assessment of plasma data than a previously published procedure and does not require the formation of diastereomers which must be maintained under exacting conditions. In addition a reliable procedure was developed that allowed for recovery of the enantiomers without affecting the ratio actually present in plasma.

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