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High-performance liquid chromatographic analysis using a highly sensitive fluorogenic reagent, 2-anthroyl chloride, and stereoselective determination of the enantiomers of mexiletine in human serum

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Abstract

A stereoselective and highly sensitive HPLC assay was developed for mexiletine enantiomers using a new fluorogenic derivatization reagent, 2-anthroyl chloride. The reagent was synthesized and utilized for the fluorescent detection (excitation at 270 nm, emission at 400 nm) of mexiletine enantiomers as their N-anthroyl derivatives on a Pirkle phenylglycine ionic HPLC column. The assay had a lower limit of quantitation at 2.5 ng/ml with a limit of detection measured at 0.5 ng/ml for each enantiomer in serum with a signal-to-noise ratio of 5:1. In a preliminary pharmacokinetic study, 200 mg of racemic mexiletine hydrochloride were administered orally to two healthy volunteers. Serum samples were collected at timed intervals over 48 h. The terminal elimination half-lives determined for total *R*(−)- and S(+)-mexiletine were 10.9 and 11.5 h, respectively. The serum free fractions for *R*(−)- and *S*(+)-mexiletine were found to be 0.56 and 0.53, respectively.

1. Introduction

Mexiletine, 1-(2′,6′-dimethylphenoxy)-2-propanamine, is a class 1b chiral antiarrhythmic agent marketed as a racemate (Mexitil). Mexiletine is orally effective for the treatment of ventricular arrhythmias with a therapeutic plasma level range of 0.7 to 2.0 μg/ml [1–4]. The plasma half-life of racemic mexiletine in healthy subjects has been reported to range from 10 to 15 h [5,6] with total serum protein binding reported to be 50–70%.

It has been well established that enantiomers differ in their stereoselective pharmacological activities and pharmacokinetics. Recent studies have shown that the pharmacological activities of mexiletine enantiomers in rats do not differ significantly [7], however, the pharmacological activities of the enantiomers in humans have not been examined. The stereoselective pharmacokinetics of the enantiomers of mexiletine in humans have been demonstrated in previous studies [8,9].

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For quantitation of mexiletine enantiomers, an HPLC method employing 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate as a chiral derivatization reagent with an assay limit of quantitation (LOQ) at 50 ng/ml was described [8]. Mexiletine and its metabolite enantiomers were also separated by reversed-phase HPLC as their *S*(+)-1-naphthyl(ethyl)isocyanate diastereomeric derivatives [10]. Although this method described the use of fluorescence detection of the naphthoyl group with a potentially low detection limit, the authors reported assay validation data only to 100 ng levels. Separation of mexiletine enantiomers following derivatization with chiral *o*-phthalaldehyde-N-acetyl-L-cysteine was described to be suitable for pharmacokinetic studies over a plasma concentration range of 2.5 to 500 ng/ml [11]. For direct separation of mexiletine enantiomers, the enantiomers were successfully separated for quantitation as their 2-naphthoyl derivatives on a Pirkle phenylglycine chiral HPLC column in our laboratory [9]. This latter method had a reported lower limit of quantitation (LOQ) at 5 ng/ml. Another approach in direct separation of mexiletine and its metabolite enantiomers as their *o*-phthalaldehyde derivatives on a Chiralpak AD stationary phase was also reported [12]. Although the derivatives were baseline resolved, the fluorescence detection of the phthalaldehyde derivatives was reported from 20 ng/ml to 2 μg/ml of plasma and urine.

To further increase the sensitivity of detection of the enantiomers, 2-anthroyl chloride was synthesized as a derivatization reagent for mexiletine enantiomers. The increase in fluorescence quantum yield of the anthroyl derivative over the naphthoyl derivative has provided a lower limit of quantitation at 2.5 ng/ml for each enantiomer compared to a limit of 5 ng/ml reported earlier [9]. The serum free (unbound) mexiletine enantiomer levels at 24 h following a therapeutic dose of 200 mg of racemic mexiletine often are in the low nanogram range and the highly sensitive assay reported in this study allowed the examination of free drug kinetics in serum of healthy volunteers.

2. Experimental

2.1. Materials

R,S-Mexiletine hydrochloride and *R,S*-mexiletine hydrochloride capsules (Mexitil), *R*(−)- and *S*(+)-mexiletine hydrochloride and the internal standard, 1-(2′,6′-dimethylphenoxy)-2-ethanamine hydrochloride, were kindly supplied by Boehringer Ingelheim (Burlington, Ont., Canada). HPLC grade *n*-hexane, diethyl ether, 2-propanol, chloroform and dichloromethane were obtained from BDH (Vancouver, Canada) as were barium hydroxide, zinc dust, concentrated ammonium hydroxide and zinc sulphate. Anthraquinone-2-carboxylic acid and oxalyl chloride were obtained from Aldrich (Milwaukee, WI, USA).

2.2. Equipment

A Gilson Model 302 liquid chromatograph, equipped with a Gilson Model 811 dynamic mixer, a Gilson Model 620 data module (Gilson Medical Electronics, Villiers Le Bel, France) and a Rheodyne 20-μl injector (Rheodyne, Berkeley, CA, USA) was used. Chromatographic flow controls were accomplished with an Apple II computer (Apple Computer, CA, USA). The HPLC column was a Pirkle 1-A phenylglycine (5 μm) 25 cm × 4.6 mm I.D. chiral column (Regis Chemicals, Morton Grove, IL, USA). An Alltech (5 μm) 15 cm × 4.6 mm I.D. silica column (Alltech Applied Science Labs., Deerfield, IL, USA) connected between the injection loop and the analytical column, was used as a guard column and to provide increased resolution of the analytes from endogenous substances. The mobile phase of isopropanol–chloroform–hexane (7:15:78, v/v) was delivered at a flow-rate of 0.8 ml/min. Fluorescence detection was optimized at excitation and emission wavelengths of 270 and 420 nm, respectively, using a Schoeffel Model GM 970 fluorometer (Kratos Schoeffel Instruments, Westwood, NJ, USA).

2.3. Synthesis of 2-anthroyl chloride derivatization reagent

The synthesis of 2-anthroyl chloride was modified from published procedures [13–15].

Reduction of anthraquinone-2-carboxylic acid with zinc/aqueous ammonia

Anthraquinone-2-carboxylic acid (0.5 g) was added to 30 ml of dilute aqueous ammonia solution (14%). The mixture was refluxed with stirring and zinc dust (2 g) was added in small portions over a 30-min period. The resulting dark red mixture, characteristic of the quinonic structure, gradually changed to a clear pale-yellow solution when the mixture was refluxed with slow stirring for 1 h. The residual zinc dust was removed by filtration and the product, 2-anthracene carboxylic acid, was precipitated by acidification of the filtrate to pH 3 with dilute hydrochloric acid. The crude product was recrystallized from hot ethanol to yield approximately 0.1 g of 2-anthracene carboxylic acid. The melting point (uncorrected) was found to be 278–279°C (lit. m.p. 276–278°C [13]).

Formation of the acid chloride

Anthracene-2-carboxylic acid (0.1 g) in 100 ml of dry benzene was added to oxalyl chloride (0.5 ml) with stirring. The mixture was slowly heated over a water bath for 30 min, after which a second 0.5-ml aliquot of oxalyl chloride was added and heating was continued for another 30 min. After cooling, the unreacted 2-anthracene carboxylic acid was filtered and removed. The excess of oxalyl chloride and solvent were removed under reduced pressure to yield 40 mg of pale-yellow 2-anthroyl chloride. The product was dissolved in a small volume of dichloromethane before precipitation from solution by the addition of hexane.

2.4. Purification of 2-anthroyl chloride by preparative-HPLC

The 2-anthroyl chloride was further purified by reversed-phase preparative-HPLC. A Hewlett-Packard Model 1082B liquid chromatograph, equipped with a Model 79850B LC terminal (Hewlett-Packard, Avondale, PA, USA), a 100-μl Rheodyne injector sample loop (Rheodyne) and a Gilson HM-Holochrome variable wavelength UV monitor (Gilson medical electronics) set at 230 nm was used. A 25 cm × 9.4 mm I.D. Whatman ODS-2 Magnum-9 column (10 μm) was used with dry acetonitrile as the mobile phase at a flow-rate of 0.7 ml/min. A 50-mg aliquot of the synthetic sample was dissolved in 1 ml of acetonitrile and 100-μl aliquots were injected onto the column. The acetonitrile column eluate corresponding to the 2-anthroyl chloride chromatographic peak at an adjusted retention time of approximately 11.0 min was collected and evaporated to dryness to yield 30 mg of 2-anthroyl chloride.

2.5. Thin-layer chromatography

A Whatman Sil-UV_{254} TLC plate (5 × 20 cm) was used with a hexane–chloroform (80:20, v/v) solvent system to evaluate the presence of any impurity in the anthroyl chloride reagent isolated by the preparative HPLC procedure. Anthroyl chloride (0.1 mg/ml) was prepared in dry chloroform and 10 μl of the solution were applied onto the plate. Following elution, the analyte was visualized as a single spot under UV light.

2.6. Derivatization of mexiletine with 2-anthroyl chloride

In a 10-ml screw-capped tube, 0.5 ml of mexiletine hydrochloride aqueous stock solution (1.0 μg/ml) was adjusted to basic pH with 200 μl of 2.0 *M* sodium hydroxide. An aliquot of 10 μl of 2-anthroyl chloride in dry dichloromethane (1 mg/ml) was added and vortex-mixed for three minutes at room temperature. Preliminary studies indicated that acylation of mexiletine was completed within three minutes. Following vortex-mixing, the anthroyl derivative of mexiletine was extracted into 2.0 ml of dichloromethane. The organic solvent was transferred to a clean tube for GC–MS analysis or evaporated to

dryness under nitrogen followed by reconstitution with HPLC mobile phase for HPLC analysis.

2.7. Structural characterization of 2-anthroyl chloride and 2-anthroyl mexiletine derivative by mass spectrometry

A Hewlett-Packard Model 5987A quadruple mass spectrometer, interfaced to a Hewlett-Packard Model 1000 data acquisition system, was used for structure identification. Samples of the N-anthroyl derivative of mexiletine and anthroyl chloride were analyzed by direct-probe MS using electron-impact ionization at 70 eV with an emission current of 300 μA and multiplier voltage of 2500 V. Source temperature was set at 240°C and probe temperature was programmed from 50°C for 1 min to 300°C for 10 min at a rate of 30°C/min.

2.8. Human pharmacokinetic study

Two healthy Caucasian male volunteers were enrolled in the study with written consent and Human Ethics approval. Physical and hematological examinations were determined to be within normal limits for the two volunteers before and after the study. Following an overnight fast (8 h), a single 200-mg dose of racemic mexiletine hydrochloride chloride (Mexitil capsule) was administered to each subject with 200 ml of water. Venous blood samples (8 ml) were collected from the forearm at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 6, 8, 10, 12, and 14 h through an indwelling Butterfly cannula (Abbott Labs, Montreal, Canada) into silicon-coated Vacutainers (Becton Dickinson, NJ, USA). The cannula was flushed with 1 ml of a sterile, isotonic heparin solution (50 IU/ml) (Allen and Hanburys, Toronto, Canada) after each blood sampling. The heparin was removed from the cannula just prior to taking the next blood sample. Additional blood samples were collected at 24, 30, 36, and 48 h by individual venipuncture. Blood samples were allowed to clot at room temperature for 2 h, then centrifuged at 2500 *g* for 15 min, and the serum separated and stored below −20°C.

2.9. Determination of mexiletine serum total (serum-protein-bound + free) and free drug concentrations

Total drug determination

In duplicate, 200-μl aliquots of serum were added to 30 μl of the internal standard solution dissolved in purified water (1 μg/ml). Serum proteins were precipitated by the addition of 200 μl of 0.3 *M* barium hydroxide solution followed by vortex-mixing, and the addition of 200 μl of 0.3 *M* zinc sulphate solution. Following protein precipitation, the serum was adjusted to alkaline pH with 200 μl of 2.0 *M* sodium hydroxide solution and extracted with two 5-ml portions of diethyl ether by vortex-mixing. The sample was centrifuged at ca. 2000 *g* for 5 min. The ethereal layer was transferred to a clean tube and reduced to ca. 1 ml under nitrogen in a 37°C water bath. An 300-μl aliquot of 0.1 *M* hydrochloric acid was added to the ether extract followed by vortex-mixing. The ethereal layer was then removed by pipette and discarded. Additionally 2 portions of 2 ml of diethyl ether were sequentially added to the aqueous layer followed by vortex-mixing and removal by pipette. The aqueous layer was adjusted to alkaline pH with 300 μl of 2.0 *M* sodium hydroxide solution and 10 μl of 2-anthroyl chloride reagent in dry dichloromethane (1 mg/ml) was added and vortex-mixed for 3 min. The mexiletine derivatives were extracted into 1 ml of dichloromethane and the organic layer was separated and reduced to dryness under nitrogen in a 37°C water bath. The samples were reconstituted in 200 μl of HPLC mobile phase prior to analysis.

Free (unbound) drug determination

Ultrafiltration of serum proteins was carried out using the Centrifree Micropartition system with a 30 000 molecular mass cut-off YMT filtration membrane (Amicon, Danvers, MA, USA). Serum pH was adjusted and maintained at pH

7.4 using buffer salts (5.2 mg of monohydrate sodium phosphate monobasic and 43.4 mg of heptahydrate sodium phosphate dibasic per ml of serum). The filtration unit was centrifuged at 1650 *g* for 20 min at 37°C using a Beckman Model Js-21 centrifuge with a 35° angle rotor head (Beckman Instruments, CA, USA). After ultrafiltration, 400 μl of the ultrafiltrate was added to 30 μl of the internal standard solution. The sample was then subjected to the barium hydroxide/zinc sulphate treatment followed by adjustment to alkaline pH and solvent extraction as described for the determination of total drug.

The non-specific binding of mexiletine in the ultrafiltration system was examined at mexiletine concentrations of 10 and 100 ng/ml prepared in serum ultrafiltrate. Following ultrafiltration, duplicate 500-μl aliquots of the serum ultrafiltrate were added to 30 μl of internal standard solution (1 μg/ml) followed by serum protein precipitation, solvent extraction, derivatization and HPLC analysis as described. Using the same procedure, duplicate aliquots of 500 μl of serum ultrafiltrate at each of the two concentrations were added to 30 μl of internal standard solution followed by sample preparation for HPLC analysis without ultrafiltration. The peak-height ratios obtained from each of the concentrations with ultrafiltration were expressed as percent of recovery of mexiletine from those obtained without ultrafiltration.

2.10. Serum total and free drug calibration curves

Serial dilutions of an aqueous stock solution of racemic mexiletine hydrochloride (1.0 μg/ml) were used to prepare duplicate serum and serum ultrafiltrate calibration samples at 100, 75, 50, 25, 12.5, and 2.5 ng/ml. An aliquot of 30 μl of an internal standard solution (1.0 μg/ml) was added to the calibration samples. Serum and serum ultrafiltrate calibration curves were constructed over the calibrated concentration range by measurement of the peak-height ratios of the mexiletine enantiomers to that of the internal standard.

2.11. HPLC assay precision and recovery

The variability in quantitation of mexiletine enantiomers between sample preparations (inter-assay variability) was studied using triplicate samples of 10 and 200 ng/ml in serum. The variability in quantitation of the enantiomers from repeated HPLC analysis of one sample (intra-assay variability) was obtained from triplicate analysis of a 10 and 200 ng/ml sample. Samples were subjected to serum protein precipitation, solvent extraction, derivatization and HPLC analysis as described.

The recovery of mexiletine enantiomers from serum and serum ultrafiltrate were studied at concentrations of 50 and 100 ng/ml. Duplicate samples at each of the concentrations were subjected to protein precipitation and solvent extraction with two 5-ml volumes of diethyl ether. Following ether extraction and re-partition in aqueous HCl, 50 ng of internal standard was added followed by derivatization and HPLC analysis. The peak-height ratios obtained were expressed as a percentage of those obtained from identical amounts of mexiletine and the internal standard directly derivatized and analyzed without prior extraction.

3. Results and discussion

The synthesis of 2-anthroyl chloride was carried out using commercially available anthraquinone-2-carboxylic acid. In this study the procedures for the synthesis of 2-anthroyl chloride were modified from a published procedure that utilized tectoquinone as the starting material [13]. In this earlier procedure, tectoquinone was used to synthesize anthracene-2-carboxylic acid and phosphorus pentachloride was used to form the acid chloride. Another report described the synthesis of 1-(1-anthryl)- and 1-(2-anthryl)-ethylamines as derivatization reagents for the separation of chiral carboxylic acids [14,15]. The authors described the synthesis of 2-anthroyl chloride from anthracene-2-carboxylic acid. However, the synthesis of anthracene-2-carboxylic acid was not described.

Reduction of substituted anthraquinone to anthracene has been reported using lithium aluminum hydride, sodium borohydride, aluminum alkoxides, zinc/ammonia solution, or hydrazine [16] with the formation of varying proportions of 9,10-dihydroxy-9,10-dihydroanthracene, 9,10-dihydroxyanthracene, anthrone, anthrol, anthracene, and other minor products. The use of zinc/ammonia solution was found to give reproducible yields of the desired anthracene-2-carboxylic acid. Formation of the acid chloride was then achieved conveniently using an excess of oxalyl chloride. The 2-anthroyl chloride was purified using reversed-phase preparative HPLC. The purity of the reagent was also ascertained using reversed-phase TLC.

Structural confirmation for both 2-anthroyl chloride and the mexiletine anthroyl derivative was obtained by direct-probe MS. The mass spectra of the reagent and the mexiletine derivative are shown in Fig. 1A and B. The [M^+] molecular ion for 2-anthroyl chloride and the mexiletine anthroyl derivative were observed at m/z 240 and 383, respectively. The mass ion at

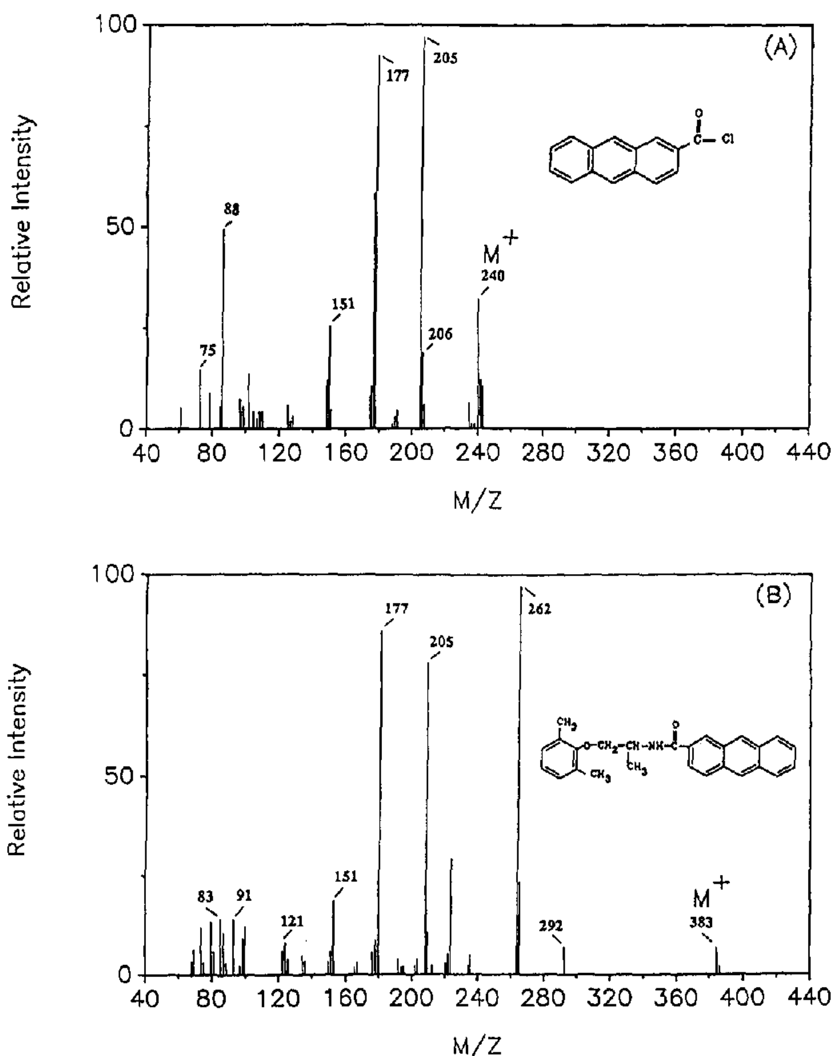

Fig. 1. Electron-impact mass spectrum of (A) 2-anthroyl chloride, and (B) mexiletine N-anthroyl derivative.

m/z 205 was common to both spectra and likely resulted from cleavage of the amide bond to give the anthroyl carbonyl fragment. The ion at *m/z* 262 likely resulted from cleavage of the xylyl ether to give the N-isopropyl anthroyl amide fragment. Other mass ions at *m/z* 151 and 177 were common to both spectra and likely resulted from the anthracene structure.

The anthroyl chloride reagent was used to develop a sensitive and stereoselective assay for the determination of mexiletine enantiomers in serum and in serum ultrafiltrate. During one of our method validation studies, a serum calibration sample was sequentially diluted to 0.5 ng/ml for each enantiomer and 10 pg of the enantiomer (injection volume of 20 μl) was detected with a signal-to-noise ratio of 5:1.

The fluorescence quantum yield (ϕ_f) of anthracene has been reported to be substantially greater than that of naphthalene [17]. This increase in fluorescence efficiency of anthracene compared to naphthalene was responsible for the observed improvement in sensitivity using the 2-anthroyl chloride reagent compared to the use of 2-naphthoyl chloride reported from an earlier study (lower detection limit, 5 ng/ml) [9]. Other examples of anthroyl derivatization reagents for HPLC fluorescence detection include the use of 1- and 9-anthroyl nitrile as sensitive derivatization reagents for HPLC analysis of hydroxysteriods [15].

One of the anthroyl derivatives of mexiletine was trapped in the detector flow cell by stopping the flow of the mobile phase. The derivative was scanned over an excitation wavelength of 200 to 600 nm. The maximal excitation wavelength was found at 270 nm. The maximal emission wavelength was similarly determined with an excitation wavelength and bandwidth of 270 and 30 nm, respectively. A bandwidth of ca. 30 nm, estimated from the spectral peak-width at half-height, was used on both the excitation and emission settings.

Derivatization of mexiletine enantiomers with 2-anthroyl chloride using the Schotten–Baumann reaction [18] conditions, catalyzed by aqueous sodium hydroxide solution, was found to be completed within three minutes. The derivatives were also found to be stable for 14 days if stored below 5°C.

Resolution of mexiletine enantiomers as their 2-anthroyl derivatives on the Pirkle 3,5-dinitrobenzoyl phenylglycine ionic HPLC chiral stationary phase (CSP) is mediated by a difference in the stability of transient diastereomeric complexes composed of π-bonding, steric interaction, electrostatic and hydrogen bonding between the enantiomers and the stationary phase [9]. These stereoselective bonding interactions between the enantiomers and the CSP are likely to be dominated by π-electron bonding between the dinitrobenzoyl group on the CSP and the anthroyl ring, amide dipole–dipole interaction between the derivative and the CSP and steric interaction of the methyl group at the chiral carbon of mexiletine, leading to the initial elution of the *R*(−)-enantiomer followed by its antipode as shown in Fig. 2.

The influence of mobile phase composition on the separation of the enantiomers was examined

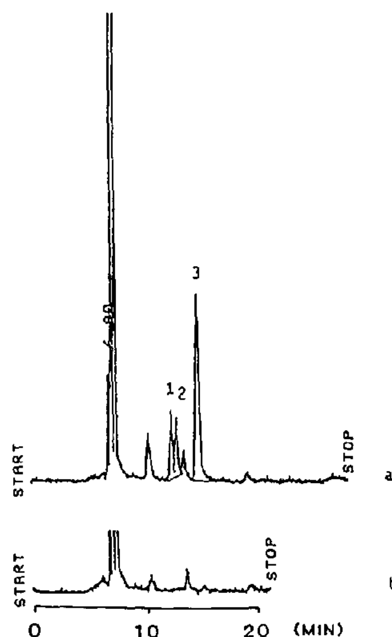

Fig. 2. Representative HPLC chromatograms showing resolution (ca. 1.3) of 10 pg of (a) N-anthroyl derivatives of *R*(−)-mexiletine (1), *S*(+)-mexiletine (2) and internal standard, KPE-2963 (3) obtained from a 5 ng/ml serum sample with 20-μl injection volume; (b) blank serum.

from a starting solvent composition of alcohol–hexane, as for most Pirkle 1A chiral columns. The composition of alcohol in the mobile phase is inversely related to the retention of mexiletine on the column. The separation factor, α, decreases with increasing isopropanol composition. Isopropanol was observed to produce better resolution of the enantiomers than methanol and ethanol, and was optimized at 7%.

The resolution, R_s, of the enantiomers was ca. 1.3 and was largely influenced by the isopropanol composition in the mobile phase. The addition of chloroform to the mobile phase improved the peak shape of the enantiomers slightly and, in addition, increased the resolution of a potentially interfering peak from the plasma extracts which eluted just after the $S(+)$ enantiomer. A silica pre-column was also connected proximal in tandem with the Pirkle chiral column which further resolved this endogenous substance from the enantiomers of mexiletine. Reduction of column temperature using a water jacket had no effect on the separation of the enantiomers. Since a column oven was not available on the LC instrument, the effect of increased temperature was not determined and all analyses were thus performed at ambient room temperature (ca. 21°C).

An attempt to identify the interference peak on the chromatogram was carried out by collecting the HPLC fraction and examining it by direct-probe GC–MS. The mass spectral data did not readily suggest a structure derived from an endogenous amine, nor did the data support a structure related to the mexiletine derivative.

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Total and free serum mexiletine calibration curves were determined over the range of 2.5 to 100 ng/ml for each of the enantiomers using 30 ng of internal standard. This assay protocol facilitated the determination of total mexiletine concentrations from 0.2 ml of serum. Free drug concentrations were determined from 0.4 ml of serum ultrafiltrate up to 48 h following administration of a single 200-mg oral dose to two human subjects. Both serum and serum ultrafiltrate calibration curves were found to be linear with a correlation coefficient (r) of >0.999. The solvent extraction efficiency of the enantiomers from serum and from serum ultrafiltrate was studied at 50 and 100 ng/ml and the mean recovery was found to be 92% (C.V. = 2.8, $n=4$) and 94% (C.V. = 2.9, $n=4$) for $R(-)$- and $S(+)$-mexiletine, respectively. Nonspecific binding of mexiletine to the ultrafiltration unit was approximately 5%. The inter- and intra-assay coefficient of variation (C.V.) was studied at 10 and 200 ng/ml serum and the overall assay variation was determined to be less than 8% as shown in Table 1.

Previous pharmacokinetic studies of mexiletine enantiomers in humans did not reveal large differences in the disposition of mexiletine enantiomers [8,9]. However the method was not sufficiently sensitive to quantitate the enantiomer concentrations of free and protein-bound drug. In order to measure the low levels of mexiletine enantiomers (<10 ng/ml) found in serum 48 h after drug administration, the stereoselective HPLC assay described in this study was developed to measure serum free drug concentrations following a single oral therapeutic dose of 200 mg of racemic mexiletine. The semilogarithmic plots of total and free $R(-)$ and $S(+)$-mexiletine concentrations in serum from one of the two subjects are shown in Fig. 3. The disposition of serum total mexiletine was best described by the pharmacokinetic computer program NONLIN [19], as a two-compartment open model with mean terminal elimination half-lives of 10.9 ± 0.4 h and 11.5 ± 0.5 h for $R(-)$- and $S(+)$-mexiletine, respectively. Serum free drug was also described by a two-compartment open model with mean terminal elimination half-lives of 8.4 ± 0.3 h and 9.3 ± 0.6 h for $R(-)$- and $S(+)$-mexiletine, respectively. The overall mean serum free fractions for the enantiomers over the 48-h period were found to be 0.56 ± 0.09 and 0.53 ± 0.09 for $R(-)$- and $S(+)$-mexiletine, respectively. The mean total drug $R(-)/S(+)$ ratio was found to decrease from 1.22 at one

Table 1
Assay variability

Mexileting serum concentration (ng/ml)	Peak-height ratio ($n = 3$)			
	Intra-assay		Inter-assay	
	$R(-)$	$S(+)$	$R(-)$	$S(+)$
10	0.16	0.15	0.16	0.15
	0.16	0.14	0.18	0..14
	0.17	0.13	0.18	0.16
Mean ± S.D.	0.16 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.15 ± 0.01
C.V. (%)	6.3	7.1	5.9	6.6
200	2.21	2.09	2.38	2.24
	2.23	2.16	2.21	2.09
	2.34	2.21	2.42	2.40
Mean ± S.D.	2.26 ± 0.06	2.15 ± 0.05	2.34 ± 0.09	2.24 ± 0.12
C.V. (%)	3.1	2.8	4.7	7.1

hour after drug administration to 0.94 at 48 h. A similar decrease in the mean $R(-)/S(+)$ ratio was observed in serum free drug.

In conclusion, the use of 2-anthroyl chloride as a derivatization reagent for the determination of mexiletine enantiomers allowed direct separation of the enantiomers on the Pirkle phenylglycine chiral HPLC column. The highly fluorescent 2-anthroyl derivative of mexiletine provided a sensitivity sufficient to determine the serum total and free drug concentrations from 0.2 ml of serum ultrafiltrate for up to 48 h in healthy subjects following oral administration of 200 mg of racemic mexiletine.

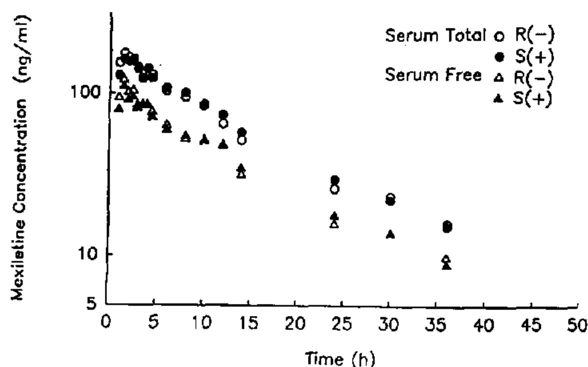

Fig. 3. Semilogarithmic plots of mexiletine enantiomer total and serum free concentrations in serum from a healthy male subject over a 48-h period following oral administration of 200 mg racemic mexiletine hydrochloride.

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