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High-performance liquid chromatographic determination of mexiletine enantiomers in plasma using direct and indirect enantioselective separations

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Abstract

Two methods were developed for the determination of mexiletine enantiomers in plasma samples suitable for studies on the stereoselective disposition of this drug. Both methods used fluorescence detection to improve sensitivity and selectivity. The direct enantioselective separation was based on the chiral resolution of mexiletine-2-naphthamide derivatives on a Chiralcel OJ column. The calibration curves were linear over the concentration range 50–500 ng/ml for each enantiomer; therefore the method can be used only for therapeutic monitoring, drug interaction and multiple dose pharmacokinetic studies. The indirect method was based on the formation of diastereomers using *o*-phthaldialdehyde and N-acetyl-L-cysteine reagents. The diastereomers were resolved on a reversed-phase RP-18 column. The method proved to be suitable for single or multiple dose pharmacokinetic studies based on the low quantification limit (1 ng/ml) and the broader linear range (1–1000 ng/ml) obtained.

Keywords: Enantiomer separation; Mexiletine

1. Introduction

Mexiletine, 1-(2,6-dimethylphenoxy)-2-aminopropane (Fig. 1), although exhibiting a chiral center in its structure, is used in clinical practice in the form of racemate for the treatment of ventricular arrhythmias [1,2].

Studies on the pharmacokinetics of this antiarrhythmic drug carried out on healthy volunteers or on patients with different pathologies are usually based on methods that do not differentiate between the enantiomers *S*-(+) and *R*-(−) [3–6]. A preliminary study [7] of the pharmacokinetics of mexiletine enantiomers in healthy volunteers has revealed significant differences in the AUC parameter (areas under the plasma concentration versus time curve) and in the percentages of renal excretion of conjugated mexiletine enantiomers. The renal excretion of the *R*-(−) enantiomer is ten-fold higher than the excretion of the corresponding *S*-(+), thus the AUC

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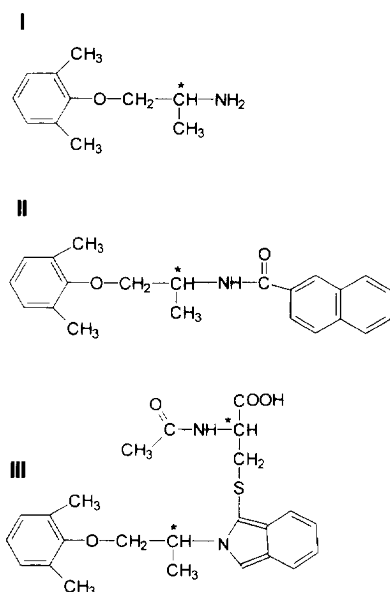

Fig. 1. Structures of mexiletine (I) and mexiletine derivatives obtained by reaction with napthoyl chloride (II) and *o*-phthalaldehyde/N-acetyl-L-cystine (III).

parameter is significantly higher for the *S*-(+) enantiomer. McErlane et al. [8] demonstrated in vitro that the percentage of binding of the *R*-(−) enantiomer to human serum proteins is significantly higher than the binding of the corresponding antipode. Based on in vitro experiments, Vandamme et al. [9] suggested that in human microsomes the aliphatic and aromatic hydroxylation of mexiletine is stereoselective. Aliphatic hydroxylation seems to be predominant for the *R*-(−) enantiomer, while aromatic hydroxylation is favored for the *S*-(+) enantiomer.

On the basis of isolated data demonstrating stereoselective kinetic disposition of mexiletine, Turgeon et al. [10] suggested that the antiarrhythmic effect of mexiletine in dogs is also stereoselective, with the *R*-(−) enantiomer showing higher activity than the corresponding *S*-(+) enantiomer.

Despite the significant clinical relevance of this topic, few studies have reported stereoselective analysis of mexiletine in plasma or serum samples.

McErlane et al. [11] have proposed the use of a Pirkle ionic column, based on *R*-(−)-3,5-dinitrobenzoylphenylglycine as chiral selector, for the separation of mexiletine enantiomers after derivatization with 2-naphthoyl chloride. Mexiletine derivatives were analyzed by HPLC, with fluorescence detection and with a sensitivity of 5 ng/ml plasma for each enantiomer. The significant interference of compounds resulting from the derivatization reaction with the analysis of low plasma mexiletine concentrations limits the application of the method to pharmacokinetic studies [12]. Another approach in direct separation of mexiletine enantiomers as their N-anthroyl derivatives on the same Pirkle phenylglycine chiral HPLC column was also reported [13]. Direct analysis of the enantiomers of mexiletine and its main metabolites in plasma and urine after their derivatization with *o*-phthaldialdehyde and 2 mercaptoethanol was developed using a chiral stationary phase based on amylose tris(3,5-dimethylphenylcarbamate), Chiralpak AD [14].

Only two studies described the use of enantiomerically pure chiral reagents for the analysis of mexiletine enantiomers as diastereomeric derivatives by HPLC from human plasma. The method described by Grech-Bélanger et al. [15] involves the preparation of diastereomers using the chiral reagent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). The diastereomeric mexiletine derivatives were analyzed by HPLC with absorbance detection and with a sensitivity of 50 ng/ml plasma, an unacceptable parameter for single-dose pharmacokinetic studies. The method of Albofathi et al. [12] is based on derivatization with *o*-phthaldialdehyde and N-acetyl-L-cysteine and fluorescence detection, with a significant improvement in the sensitivity and selectivity of the analytical method.

Other chiral derivatization reagents have been used in indirect methods for the separation of mexiletine enantiomers as diastereomeric derivatives from different matrices. Freitag et al. [16] described a stereospecific method for mexiletine and its two major metabolites isolated from microbial fermentation media using pre-column derivatization with *S*-(+)-1-(naphthyl) ethyl isocyanate followed by normal-phase HPLC. In urine samples the enantiomeric ratios of mexiletine were determined by gas–liquid chromatography and flame ionization detection as their N-trifluoracetyl-*S*-(−)-prolyl chloride diastereomeric derivatives [17].

The present study compares procedures for the analysis of mexiletine enantiomers in human plasma

using a chiral stationary phase (direct method) and an achiral stationary phase after conversion of the enantiomers to diastereomeric derivatives (indirect method), and establishes confidence limits compatible with studies of drug interaction and single-dose pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

A stock solution of mexiletine (100% mexiletine hydrochloride, kindly supplied by Boehringer De Angeli, Brazil) was prepared in water at the concentration of 100 μg free base/ml. Working standards were prepared in water at the concentrations of 0.2, 1.0, 2.0, 5.0 and 10.0 μg/ml for each enantiomer. The solutions were stored at −20°C and were stable for at least three months.

The derivatization reagents were obtained from Aldrich (98% 2-naphthoyl chloride) and Sigma (99% *o*-phthaldialdehyde and 99% N-acetyl-L-cysteine). The solvents used in the extraction and derivatization procedures and in the chromatographic analysis were p.a. or chromatography grade.

2.2. Extraction procedure

A 500-μl plasma sample was alkalinized with 50 μl of a 0.3 *M* aqueous sodium hydroxide solution (previously washed with di-isopropyl ether), then extracted twice with 4.0 ml di-isopropyl ether by vortex-mixing for 2 min. After centrifugation at 1800 *g* for 10 min and separation of the organic phase, the combined extracts were supplemented with 50 μl 0.1 *M* HCl (prepared in methanol) and evaporated to dryness under a nitrogen flow.

2.3. Derivatization procedures and chromatographic analysis

2.3.1. Direct method

The residue obtained in the extraction procedure was dissolved in 100 μl of 0.1 *M* aqueous HCl solution (previously washed with di-isopropyl ether), 100 μl of 2 *M* aqueous NaOH solution (previously washed with di-isopropyl ether) and 200 μl of water. After vortex-mixing for 15 s and a rest of 5 min, mexiletine was derivatized with 100 μl of a 2-naphthoyl chloride solution prepared in dichloromethane at the concentration of 4 mg/ml. After vortex-mixing for 2 min, the compounds *R*-(−)-mexiletine-2-naphthamide and *S*-(+)-mexiletine-2-naphthamide (Fig. 1) were extracted from the alkaline aqueous phase with 2 ml of the hexane–isopropanol mixture (9:1, v/v) by vortex-mixing for 2 min, followed by centrifugation at 1800 *g* for 5 min. The organic phase was evaporated to dryness under a nitrogen flow and the residues were dissolved in 200 μl of the hexane–isopropanol mixture (9:1, v/v).

Separation of the enantiomers of mexiletine derivatized with 2-naphthoyl chloride was carried out on a chiral stationary phase based on cellulose tris(*p*-methylbenzoate), a Chiralcel OJ column, 250×4.6 mm, 10 μm particles, protected by a 50 mm Chiralcel OJ guard column (Chiral Technologies, Exton, PA, USA). A CG Model 480 C liquid chromatography apparatus (São Paulo, Brazil) was used with a 10-μl loop, a fluorescence detector (Shimadzu Model RF 535) operating at 230 nm (λ_{exc}) and 340 nm (λ_{em}) and a Varian Model 4270 integrator. The mobile phase consisted of a mixture of hexane–ethanol (7.1:2.9, v/v) at a flow-rate of 1 ml/min.

2.3.2. Indirect method

The residue obtained in the extraction procedure was dissolved by the addition of 25 μl of 0.03 *M* aqueous HCl and 50 μl of 0.1 *M* aqueous sodium borate. The derivatization reaction (Fig. 1) was carried out by the addition of 100 μl of the chiral reagent (4 mg *o*-phthaldialdehyde and 5 mg N-acetyl-L-cysteine dissolved in 0.5 ml methanol) and vortex-mixed for 30 s.

For analysis of mexiletine enantiomers derivatized with the chiral reagent we used a Varian Model 5000 liquid chromatography apparatus equipped with a Rheodyne injector (20-μl loop), a Shimadzu Model RF-535 fluorescence detector using excitation and emission wavelengths fixed at 350 nm and 455 nm, respectively and a Varian Model 4270 integrator. The diastereomers were separated on a reversed-phase LiChrospher 100 RP-18 column (150×4 mm, 5 μm particles, Merck), with a guard column (4×4 mm) of the same material. The mobile phase consisted of a

mixture of methanol and 0.05 *M* acetate buffer, pH 5.5, at the 6.5:3.5 proportion (v/v) and a flow-rate of 1 ml/min.

2.4. Calibration curves and validation of the methods

The calibration curves were obtained by the analysis (in duplicate) of 500-μl blank plasma samples (obtained from volunteers not treated with mexiletine) supplemented with 25 μl each of working standard mexiletine solutions. The concentration ranges of the enantiomers were 50–500 and 10–500 ng/ml plasma, respectively, for the direct and indirect methods. The samples were then submitted to the procedures of extraction, derivatization and chromatographic analysis described earlier.

For validation of the methods we calculated recovery, linearity, precision, sensitivity and selectivity. It should be pointed out that all parameters, as well as the calibration curves were determined after the addition of the two major metabolites of mexiletine, i.e., hydroxymethylmexiletine (hydroxymethylmexiletine oxalate, KOE 2259) and *p*-hydroxymexiletine (*p*-hydroxymexiletine hydrochloride, KOE 2127), kindly supplied by Boehringer Ingelheim, at concentrations 50% lower than those of mexiletine.

The recovery of mexiletine enantiomers from plasma was studied at concentration ranges of the calibration curves. The recovery was obtained by comparing the peak heights obtained after derivatization of mexiletine extracted from plasma with peak heights obtained after derivatization of the same amount of unextracted mexiletine.

The quantification limit was defined as the lowest plasma concentration of mexiletine enantiomers quantified with a coefficient of variation of less than 10%.

The precision of the method was examined at two different concentrations of mexiletine (60 and 500 ng/ml plasma of each enantiomer for the direct method and 10 and 280 ng/ml plasma of each enantiomer for the indirect method). The results were expressed as the intra-day ($n=10$) and inter-day ($n=5$) coefficients of variation.

The selectivity of each method was evaluated after derivatization of the drugs at concentrations within the therapeutic interval.

2.5. In vivo study

The methods for the analysis of mexiletine enantiomers in plasma were applied to the investigation of the stereoselective kinetic disposition of mexiletine administered orally to a healthy volunteer.

The volunteer, a 37-year old male weighing 65 kg and 1.65 m tall, was informed about the study and gave written consent to participate. After clinical examination and biochemical tests for the confirmation of normal hepatic, renal and cardiac function, the volunteer received two capsules of Mexitil (100 mg, Boehringer De Angeli, Brazil) in the morning after a 12-h fast. Breakfast was served 3 h after the administration of the drug. Serial blood samples were drawn via a heparinized intravenous catheter at times 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 30, 36 and 48 h after Mexitile administration. The blood samples were transferred to tubes containing heparin (Liquemine, 5000 IU, Roche) and centrifuged at 1800 *g* for 10 min. The plasma samples were stored at −20°C until chromatographic analysis.

3. Results and discussion

The direct resolution of mexiletine enantiomers was carried out on a Chiralcel OJ column after derivatization with 2-naphthoyl chloride, which in basic medium reacts with mexiletine forming the fluorescent mexiletine-2-naphthamide [11]. At the end of the reaction, the derivatives were extracted from the alkaline aqueous phase with an organic solvent compatible with injection into the chiral column. Fig. 2 shows the resolution of derivatized mexiletine enantiomers and the resolution of enantiomers of the major metabolite detected in plasma, hydroxymethylmexiletine. The more polar metabolite *p*-hydroxymexiletine was not eluted under the conditions used. Elution of the derivatized mexiletine enantiomers in the *R*-(−) and *S*-(+) sequence was established on the basis of injection of each enantiomer obtained according to the procedure described by Turgeon et al. [10].

The mexiletine enantiomers in plasma samples

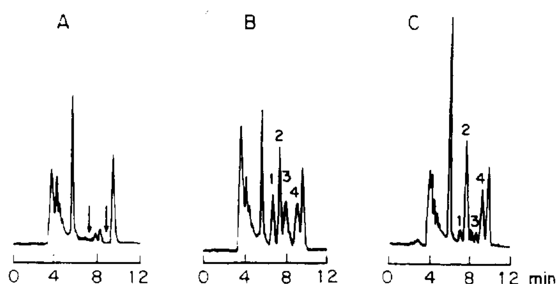

Fig. 2. Analysis of plasma mexiletine enantiomers using the direct method. The chromatograms refer to samples of (A) blank plasma; (B) plasma supplemented with *p*-hydroxymexiletine (ND), hydroxymethylmexiletine (1,3), I-*R*-(−)-mexiletine (2) and *S*-(+)-mexiletine (4); and (C) plasma from a volunteer treated with mexiletine.

were also analyzed by an indirect method following derivatization with the chiral reagent *o*-phthaldialdehyde/N-acetyl-L-cysteine [12]. Fig. 3 demonstrates the separation of the mexiletine enantiomers from the enantiomers of the two major metabolites hydroxymethylmexiletine and *p*-hydroxymexiletine detected in plasma. No interference from reagents or endogenous plasma components was observed. The derivatized mexiletine enantiomers were eluted in the *S*-

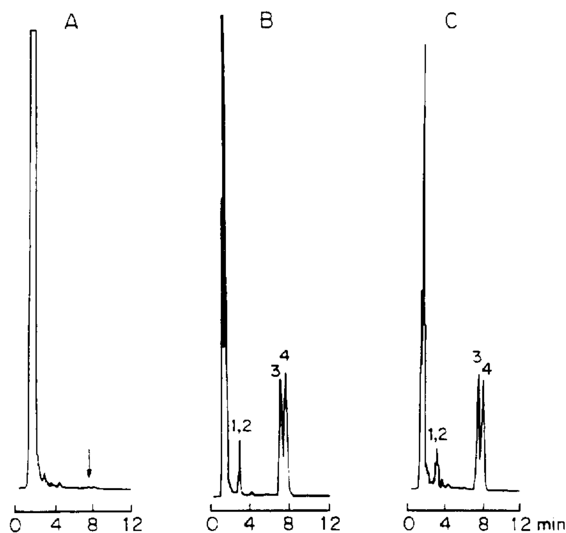

Fig. 3. Analysis of plasma mexiletine enantiomers using the indirect method. The chromatograms refer to samples of (A) blank plasma; (B) plasma supplemented with *p*-hydroxymexiletine and hydroxymethylmexiletine (1,2), *S*-(+)-mexiletine (3), and *R*-(−)-mexiletine (4); and (C) plasma from a volunteer treated with mexiletine.

(+) and *R*-(−) sequence on the basis of injection of each enantiomer separately, according to the study of Albofathi et al. [12].

Table 1, Table 2, and Table 3 show the confidence limits obtained in the validation of the methods.

The data in Table 1 demonstrate that the recovery of mexiletine enantiomers in plasma samples was more than 90% and was independent of the concentration in the range used for the calibration curves. The values obtained exceed the 83% recovery reported by McErlane et al. [11] in a study in which plasma proteins were precipitated with zinc sulfate and barium hydroxide before the extraction procedure. Direct plasma extraction at pH 10.4 with two portions of di-isopropyl ether, in addition to providing excellent recovery, resulted in chromatograms free of endogenous plasma interferents in the region of interest (Fig. 2 and Fig. 3).

In the direct method, the linearity was narrow, with a range of 50–500 ng/ml, due to the amount of 2-naphthoyl chloride employed in the derivatization reaction. The linearity can be increased by increasing the amount of 2-naphthoyl chloride, but always with impairment of the quantification limit due to the fact that the derivatization reaction results in a peak coeluted with the enantiomer *R*-(−)-mexiletine. Interference was minimized up to the addition of 0.4 mg 2-naphthoyl chloride to the 0.5 ml plasma extract, a quantity compatible with the quantification limit of 50 ng/ml. In contrast, the indirect method presented a broader linear range of 1–1000 ng/ml with a highly significant reduction of the limit of quantification.

The linearity of the methods was determined in the presence of the two major metabolites detected in plasma at concentrations equivalent to 50% of the unchanged drug. This procedure is essential because the metabolites also present the primary aliphatic amine group and therefore also consume the derivatization reagents. McErlane et al. [11], using the same non-chiral reagent and elution of mexiletine derivatives on a Pirkle ionic column observed linearity in the 5–750 ng/ml plasma range with the use of the reagent in amounts approximately 40 times lower. The method described by Abolfathi et al. [12] was linear over the concentration range 2.5–500 ng/ml plasma for each enantiomer but

Table 1
Confidence limits of the methods for analysis of mexiletine enantiomers in plasma samples

	Recovery (%)	Limit of quantification (ng/ml)	Linearity (ng/ml)
Direct method			
R-(−)-Mexiletine	90.3	50.0	50.0–500.0
S-(+)-Mexiletine	93.8	50.0	50.0–500.0
Indirect method			
R-(−)-Mexiletine	91.3	1.0	1.0–1000.0
S-(+)-Mexiletine	90.5	1.0	1.0–1000.0

using two standard curves. Neither method mentions the metabolites present in plasma.

The precision of the method was evaluated on the basis of intra-day and inter-day precision. The data in Table 2 demonstrate coefficients of variation of 10% or less, a parameter that shows the reliability of the results in the 50 to 500 ng/ml plasma concentration range for the direct method and in the 1 to 1000 ng/ml range for the indirect method.

The data obtained in the validation study also show that the use of an internal standard is not necessary for these analyses. The option to use only external standards was based on avoiding the consumption of the derivatization reagent and on expanding as much as possible the linear range of plasma concentrations of the mexiletine enantiomers.

In the selectivity study of the method we evaluated approximately twenty drugs that could be administered in combination with mexiletine. The high selectivity of the methods presented in Table 3 confirms that chemical derivatization, in addition to significantly increasing the detectability of mexiletine, also contributes to the improvement of the overall selectivity of the method, as some of the drugs tested are not fluorescent or do not react with the fluorescent reagents. In addition, it may be possible that some drugs not detected did not elute from the column.

The direct and indirect methods were employed in the analysis of samples obtained from a healthy volunteer after administration of a single dose of 200 mg mexiletine hydrochloride. The direct method permitted the quantification of the plasma concentrations of both mexiletine enantiomers only up to 10 h after administration, a fact that impairs the application of the method to the study of single dose kinetic

Table 2
Precision of the methods for analysis of mexiletine enantiomers in spiked plasma samples

	Direct method				Indirect method			
	R-(−)-Mexiletine		*S*-(+)-Mexiletine		*R*-(−)-Mexiletine		*S*-(+)-Mexiletine	
Intra-day								
$\bar{X}$ (ng/ml)	62.1	484.2	62.9	464.6	9.0	278.0	9.4	282.0
n	10	10	10	10	10	10	10	10
C.V. (%)	9.1	4.2	9.3	3.7	2.6	4.7	3.1	4.9
Inter-day								
$\bar{X}$ (ng/ml)	59.2	500.2	63.1	461.4	10.6	273.0	10.2	277.6
n	5	5	5	5	4	4	4	4
C.V. (%)	10.0	4.4	10.0	5.1	4.9	4.4	4.2	3.2

$\bar{X}$, mean concentration; *n*, number of samples analyzed; C.V., coeffcient of variation.

Table 3
Study of the selectivity of the methods for analysis of mexiletine enantiomers using direct and indirect separation

Drug	Concentration (ng/ml)	Retention time (min)	
		Direct method	Indirect method
p-Hydroxymexiletine (1)	250	ND	2.3
p-Hydroxymexiletine (2)	250	ND	2.7
Hydroxymethylmexiletine (1)	250	6.6	2.7
Hydroxymethylmexiletine (2)	250	7.8	2.9
S-(+)-Mexiletine	500	8.9	7.4
R-(−)-Mexiletine	500	7.2	8.0
Ammopyrine	1000	-	ND
Amiodarone	750	ND	ND
Benzidamine	800	-	ND
Captopril	490	ND	ND
Carbamazepine	1200	-	ND
Clobazam	950	ND	ND
Clomipramine	70	-	ND
Clonazepan	70	ND	ND
Chlorpromazine	122	-	ND
Dapsone	1440	-	1.4
Digoxin	2.5	-	ND
Disopyramide	500	ND	ND
Ergotamine	4	ND	-
Ethidocaine	1000	-	ND
Lidocaine	5000	-	ND
Metoclopramide	130	-	ND
Nitrazepan	70	ND	-
Nor-disopyramide	990	ND	-
Pindolol	80	ND	-
Procainamide	10 000	-	ND
Propafenone	600	ND	ND
Propranolol	1000	ND	ND
Quinidine	6000	-	4.5
Sotalol	1400	ND	ND
Theophylline	15 000	-	ND
Trimipramine	240	-	ND
Verapamil	68	-	ND

ND, Not detected; - Not injected; 1 and 2 refer to the first and the second enantiomer eluted, respectively.

disposition because it prevents the characterization of the slow-decay phase. In addition, in the direct method mexiletine enantiomers are likely to suffer greater interference. This is particularly important for enantiomer *R*-(−) which is eluted between the two enantiomers of the hydroxymethylmexiletine metabolite (Fig. 2).

The indirect method permitted the quantification of the *S*-(+) and *R*-(−) enantiomers of mexiletine 48 h after oral administration of the antiarrhythmic drug (Fig. 4). The decay curve of the plasma concentrations as a function of time (log C versus t) characterizes a bicompartmental model with an elimination half-life (slow-decay phase) of 10 h for both enantiomers. The AUC parameter, which characterizes the amount of the drug in the biological system, was calculated by the trapezoidal rule and indicated a larger amount of the *S*-(+)-enantiomer ($AU_{0 \rightarrow 48}$=1344.1 ng h ml^{-1}) than of the *R*-(−)-enantiomer ($AUC_{0 \rightarrow 48}$=1200.5 ng h ml^{-1}). These data agree with those reported by Grech-Bélanger et al. [7].

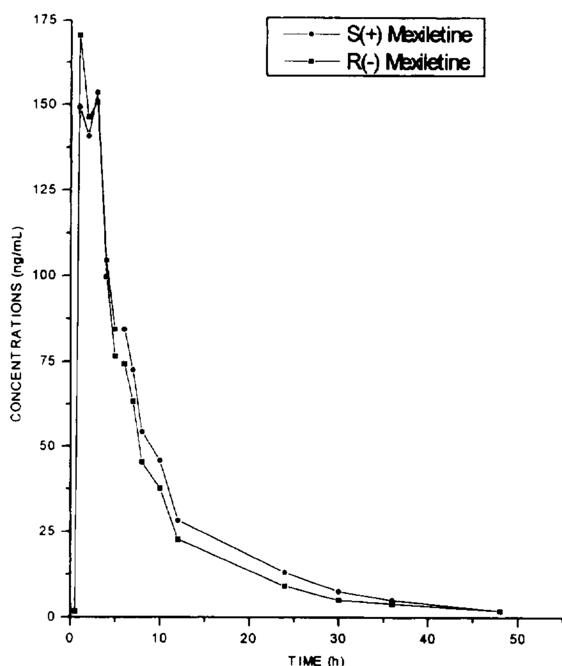

Fig. 4. Plasma concentration–time curve for S-(+)-mexiletine and R-(−)-mexiletine (indirect method) in one volunteer.

The confidence limits obtained in the validation of the indirect method demonstrate that incomplete resolution of the enantiomers did not result in significant errors. In the analysis of plasma samples obtained from kinetic disposition studies the enantiomers were present at approximately equal concentrations, thus reproducing the conditions used for method validation, in which racemic mexiletine was used. This procedure has several advantages when compared to the method of Albofathi et al. [12], which employs the same derivatization reagent. A smaller volume of plasma (500 μl) is used and analysis time is reduced by 50%, highly relevant conditions in studies of kinetic disposition requiring serial blood collections. Furthermore, a quantification limit of 1 ng/ml was obtained together with a greater linear concentration range (1–1000 ng/ml) than in the study previously reported. These conditions favor the application of the method to studies of kinetic disposition of a single dose, multiple doses and therapeutic monitoring. We should finally point out the high selectivity of this method which favors its application to interaction studies.

4. Conclusions

Two methods were developed for the separation of mexiletine enantiomers in plasma samples and validated for use in a study of enantioselective disposition of mexiletine.

To our knowledge, the column used in the direct method has not been employed before for the resolution of derivatized mexiletine enantiomers. The method has the advantages of using only 500 μl plasma, not requiring the step of plasma protein precipitation, and providing higher recovery of the R-(−)- and S-(+)-enantiomers. Furthermore, it allows the separation of the enantiomers of mexiletine from those of the major metabolites detected in plasma, and the elution of the enantiomers occurs over a time 50% shorter than that observed with the Pirkle-type column. Finally, no interference of endogenous components or of other associated drugs was observed. However, the quantification limit of 50 ng/ml and the linear range of 50–500 ng/ml prevent the application of the method to single dose pharmacokinetic studies.

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