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Assay of tocainide enantiomers in plasma by solid-phase extraction and indirect chiral high-performance liquid chromatography after derivatization with (−)-menthyl chloroformate

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Abstract

A fast high-performance liquid chromatographic (HPLC) assay was developed for determination of tocainide enantiomers in plasma. Subsequent to solid-phase extraction of tocainide from plasma, homochiral derivatization with (−)-menthyl chloroformate enabled separation of the enantiomers by a conventional reversed-phase HPLC system. The detection was performed by UV absorption at 262 nm. An enantiomeric resolution of 1.0 was obtained. Linearity of the method was investigated and found to be good in the range from 1.0 to 20.0 μg/ml tocainide enantiomer and the limit of quantitation was 1.0 μg/ml. The method was applied to a study of the distribution and elimination pharmacokinetics of tocainide enantiomers in the rabbit. No difference in distribution or elimination between the enantiomers was found nor did the enantiomers affect the disposition of one another when administered together as the racemate.

1. Introduction

Tocainide (2-amino-N-(2,6-dimethylphenyl)-propanamide) is a congener of lidocaine for oral treatment of ventricular arrhythmias [1]. Tocainide possesses a chiral centre and is clinically used as the racemate. Due to potential proarrhythmic effects [2], therapeutic drug monitoring of plasma concentrations of tocainide may be necessary. Since the human elimination of tocainide is stereoselective [3,4], the analytical method must be able to discriminate between the enantiomers.

In spite of the rapid development in chiral stationary phases for HPLC, the indirect approach to enantioselective analysis still has merits for assays in biological samples due to the better selectivity generally obtained by non-chiral reversed-phase stationary phases [5].

Several HPLC methods based on the indirect approach for the determination of tocainide enantiomers in biological samples have been

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published. Separation by normal-phase chromatography has been achieved following derivatization with *S*-(+)-1-(1-naphthyl)ethyl isocyanate [6] and *R*-(−)-O-methyl mandelic acid chloride [7]. The former had a derivatization yield of less than 50% and the second was only used for determination of the enantiomeric ratio in urine samples. Using reversed-phase chromatography, Gal et al. have separated the enantiomers of tocainide as thiourea derivatives of several chiral isothiocyanate reagents [8] from which *S*-1-(1-naphthyl)ethyl isothiocyanate was used for a study of the pharmacokinetics of the enantiomers in plasma and urine.

The same research group used tocainide as a model substance in a study of the separation of chiral primary amines by *o*-phthalaldehyde (OPA)–homochiral thiol derivatization [9]. This approach was not implemented for biological samples.

Although better detection limits may be achieved by use of fluorescent derivatizing agent, the excess reagent has to be removed [6,8] as part of the sample preparation procedure to avoid interference with the chiral HPLC separation. This is not a problem with the OPA method since the reagent is non-fluorescent but the resulting diastereomeric isoindole derivatives showed different detector responses [9].

Chloroformates react under mild conditions with both primary and secondary amines and hydroxyl groups forming stable derivatives [10]. (−)-Menthyl chloroformate is derived from the natural product (−)-menthol and possesses high chiral purity. It is UV transparent and thus it should not affect the UV response of the enantiomers [11]. Furthermore, this chiral derivatizing agent is commercially available at a moderate price. For HPLC, (−)-menthyl chloroformate has primarily been used for separations of the enantiomers of β-adrenergic antagonists [12,13] including an assay for atenolol enantiomers in human plasma and urine [14].

This paper describes a fast HPLC assay for determination of tocainide enantiomers in plasma utilizing solid-phase extraction and derivatization with (−)-menthyl chloroformate.

2. Experimental

2.1. Chemicals

S-(+)-, *R*-(−)- and racemic tocainide in form of the hydrochlorides (*S*-toc, *R*-toc and rac-toc) were donated by Astra Hässle (Mölndal, Sweden).

(−)-Menthyl chloroformate and benzylamine hydrochloride (I.S.) was obtained from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC-grade and were used throughout the investigations.

Potassium phosphate stock solutions (0.2 *M*) of pH 5.0 and 10.0 were used for preparing the mobile phase and the solid-phase extraction wash buffer, respectively.

2.2. Reagent and standard solutions

The derivatizing agent solution was prepared by diluting 1.1 g of (−)-menthyl chloroformate in 5 ml (1 *M*) of acetonitrile. When refrigerated, this solution could be kept for several months.

To prepare spiked plasma samples a stock solution of 10.00 mg of rac-toc in 10.0 ml of water was made.

The I.S. stock solution contained 1.2 mg/ml benzylamine hydrochloride in water.

2.3. Instrumentation

The analytical HPLC-system consisted of a WISP 710A autosampler (Millipore, Milford, MA, USA), set at an injection volume of 20 μl and a LC-6A liquid chromatograph equipped with a SPD-6A variable wavelength UV detector (Shimadzu, Kyoto, Japan) operating at 262 nm. Chromatograms were recorded on a DP 700 data processor (Carlo Erba, Valencia, CA, USA) and the peaks were quantified by height measurement.

For comparison of the UV spectra of the two diastereomeric derivatization products diode-array detection was performed using a 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA). UV spectra in the wavelength

range 220–300 nm were recorded at the apex of the peaks.

2.4. Chromatography

The separation was performed at ambient temperature using a 120 × 4.6 mm I.D. column (Knauer, Berlin, Germany) packed with Spherisorb ODS-2, 5-μm particles (Phase Separation, Queensferry, UK). The mobile phase was composed of methanol, 0.2 *M* potassium phosphate pH 5.0 and water (30:1:9, v/v/v) and delivered at a flow-rate of 1.0 ml/min.

2.5. Solid-phase extraction procedure

Plasma samples were cleaned by means of Bond Elut 100 mg C_{18} extraction cartridges worked by a Vac Elut system (Varian, Harbor City, CA, USA).

To reduce the amount of coextracted plasma substances, each sample was alkalized to pH 11 with ca. 100 mg of solid sodium carbonate and whirli-mixed prior to extraction.

Initially the cartridges were rinsed with 1 ml of methanol and 1 ml of 20 m*M* potassium phosphate wash buffer pH 10. A 1-ml aliquot of plasma sample containing 25.0 μl of I.S. solution was then applied to the cartridge. Before the final elution with 0.5 ml of methanol the cartridges were rinsed with 1 ml of wash buffer. The methanolic eluates were collected in autosampler vials.

Without deterioration of the method reproducibility, the cartridges could be used for clean up of several samples when the rinsing procedure was repeated before each re-use.

2.6. Derivatization procedure

The derivatization reaction is shown in Fig. 1. To the methanolic eluate was added 20 μl of 1 *M* (−)-menthyl chloroformate solution in acetonitrile. The vial was shaken briefly and left for 3 min before chromatography.

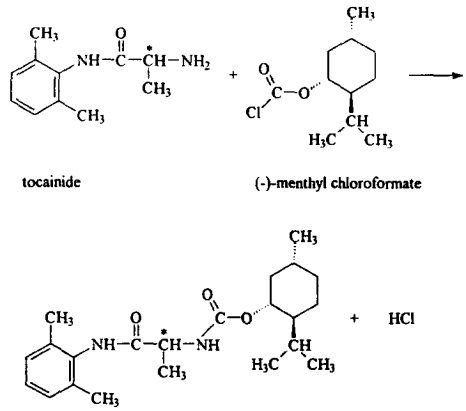

Fig. 1. Reaction between tocainide and (−)-menthyl chloroformate.

2.7. Extraction and derivatization yield

Determination of the recovery of the solid-phase extraction procedure was carried out with aqueous solutions of racemic tocainide (20.0 μg/ml) and benzyl amine (20.0 μg/ml). The amount of tocainide or I.S. in the waste water, the wash buffer and the methanolic eluate was measured by HPLC and compared with methanolic standards (20.0, 40.0 and 80.0 μg/ml). The underivatized substances had retention times of 1.3 and 1.5 min for tocainide and benzyl amine, respectively.

To determine the derivatization time, the derivatization procedure was carried out for 0.5 ml of methanolic solutions of racemic tocainide (40.0 μg/ml) or benzyl amine (40.0 μg/ml) to which was added a drop of solid-phase extraction wash buffer pH 10. The reaction was followed for 15 min by HPLC analysis.

2.8. Animal study

Four male albino rabbits weighing 2545 g ± 102 (S.D.) were given racemic tocainide hydrochloride 25.0 mg/kg and each of the enantiomers 12.5 mg/kg on crossover basis with an interval of one week between doses. The drugs were administered as bolus injections in an ear vein,

whereupon blood samples were collected from another ear vein into heparinized tubes at 5, 15 and 30 min and 1, 2, 4, 6, 8 and 24 h. The samples were centrifuged and the separated plasma was stored at −20°C until analysis.

2.9. Treatment of data

The peak-height ratio of toc/I.S. was used to determine the concentration of each enantiomer.

A two compartment model, $C_{pl}(t) = Ae^{-\alpha t} + Be^{-\beta t}$, was best fitted to the drug concentration–time data from the animal study. The volume of distribution of the central compartment was calculated as Vd_1 = i.v. dose/$(A + B)$ and the volume of distribution in steady-state as Vd_{SS} = i.v. dose (AUMC)/(AUC)^2. The body clearance was determined by Cl_B = i.v. dose/(AUC). AUC is the total area under the curve and AUMC is the total area under the first moment curve. The fitting was performed by use of the Simplex procedure [15] written in a programme adopted from Yamaoka et al. [16]. Throughout the fittings, the reciprocal of the concentration squared was used as a weighting factor.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of plasma samples are shown in Fig. 2. From chromatography of derivatives of the single enantiomers *R*-tocainide menthyl carbamate was determined to be the least retained diastereomer. Retention times were 4.70, 5.07 and 6.22 min for the derivatives of *R*-toc, *S*-toc and the internal standard, respectively.

The enantiomeric peak resolution was 1.0, which is not baseline separation. Increasing the retention by decreasing the amount of methanol in the mobile phase did not improve the resolution, nor did changing the pH of the mobile phase in the range from 5 to 7. When substituting acetonitrile or tetrahydrofuran for methanol, the derivatives were not separated at all using the C_{18} material.

Better resolution (R_s) of tocainide enantiomers in reversed-phase systems have been obtained by thiourea derivative formation, R_s = 2.05 to 3.14 [8], and by isoindole derivative formation, R_s = 1.64 to 2.00 [9].

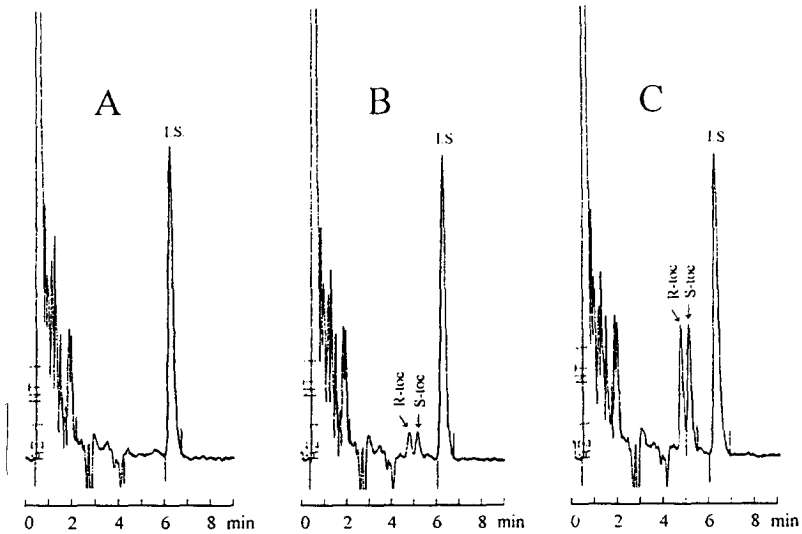

Fig. 2. Chromatograms of blank plasma spiked with 30 μg/ml internal standard. (A) Plasma blank, (B) 2.0 μg/ml, and (C) 10.0 μg/ml racemic tocainide hydrochloride.

3.2. Extraction and derivatization

The evaluation of the extraction procedure showed no tocainide or benzyl amine in the waste water nor in the wash buffer. For both substances the concentration found in the methanolic eluate showed the expected two-fold concentration relative to the original sample, corresponding to 100% recovery in the extraction step.

To achieve a final sample solution with a composition resembling the mobile phase of the HPLC system, extraction eluents of methanol–water (90:10, v/v, or 80:20, v/v) were examined, but less than 100% recovery was obtained with these eluents.

The derivatization reaction of (−)-menthyl chloroformate with tocainide was instantaneous under the described conditions. No remnant of underivatized tocainide could be detected in the reaction mixture immediately after addition of the reagent, and when re-analysed after 15 min, the response of the derivatives had not increased. The derivatization of benzyl amine was examined in a similar manner and found to be completed within 3 min.

The high recovery of the extraction procedure and the fast time course of the derivatization reaction should prevent changes in the relative ratio of the enantiomers prior to chromatography.

The methods previous published on simultaneous determination of tocainide enantiomers in biological samples [6,8] were based on liquid–liquid extraction which is a time-consuming process relative to solid-phase extraction. On the other hand, a greater degree of concentration of the samples was obtained during liquid–liquid extraction, which was reflected in the lower limit of detection (0.25 μg/ml) achieved by these methods.

3.3. Calibration curves

Linearity of the method was determined in the range of 1.0–20.0 μg/ml tocainide enantiomer. The lower limit of quantitation (LOQ) was set at 1.0 μg/ml (equivalent to 40 ng tocainide enantiomer injected), corresponding to the lowest concentration to be determined with acceptable accuracy and precision.

The UV spectra of the diastereomeric derivatives were determined to be identical when compared using the diode-array detector software. In accordance, the calibration curves for the individual diastereomers were identical within 95% confidence, $y = (-0.004 \pm 0.054) + (0.089 \pm 0.005)x$ for *R*-(−)-tocainide and $y =$

Table 1
Intra- and inter-day accuracy and precision of the method

Added (μg/ml)	*R*-Tocainide			*S*-Tocainide		
	Found (μg/ml)	Recovery (%)	C.V. (%)	Found (μg/ml)	Recovery (%)	C.V. (%)
Intra-day (n = 7)[a]						
2.50	2.43 ± 0.18	97.2	7.5	2.27 ± 0.15	90.6	6.7
5.00	5.06 ± 0.09	101.1	1.8	5.11 ± 0.10	102.1	2.0
15.00	14.33 ± 0.49	95.5	4.9	14.30 ± 0.49	95.3	3.4
Inter-day (n = 9)[b]						
1.03	1.20 ± 0.15	116.5	12.2	1.19 ± 0.14	115.5	11.5
5.15	4.98 ± 0.35	96.6	7.1	4.97 ± 0.37	96.5	7.3
10.30	10.61 ± 0.91	103.0	8.5	10.57 ± 0.93	102.6	8.8

[a] Added in the form of 5.00, 10.00 and 30.00 μg/ml racemic tocainide hydrochloride, respectively.
[b] Added in the form of 2.06, 10.30 and 20.60 μg/ml racemic tocainide hydrochloride, respectively.

$(-0.007 \pm 0.059) + (0.088 \pm 0.005)x$ for *S*-(+)-tocainide ($n = 3$) with correlation coefficients, r^2, of 0.9985 and 0.9982, respectively.

3.4. Accuracy and precision

The recovery and the coefficient of variation of the method at three concentration levels are given in Table 1.

The slight overestimation of the lower-concentration samples can be ascribed to the incomplete peak resolution which causes a slight negative intercept of the calibration curve.

3.5. Animal study

In humans, the principal metabolite of tocainide is the carbamoyl O-β-D-glucuronide, which accounts for 20–30% of the elimination of a racemic tocainide dose [17]. This metabolic reaction is stereoselective, with *R*-tocainide being conjugated to a far greater extent than *S*-tocainide [4]. As this metabolic pathway is of quantitative importance in humans only [18], there is still a lack of an animal model for the stereoselective disposition of tocainide. The same study [18] showed that in eight animal species, acid hydrolyzable conjugates of tocainide were produced to the highest extent (13% of a tocainide dose) in the rabbit. This study was undertaken to determine whether the disposition of tocainide in the rabbit is stereoselective.

Apart from rabbit No. 4, where one of the profiles differed considerably from the others, no difference in distribution or elimination between the enantiomers was found nor did the enantio-

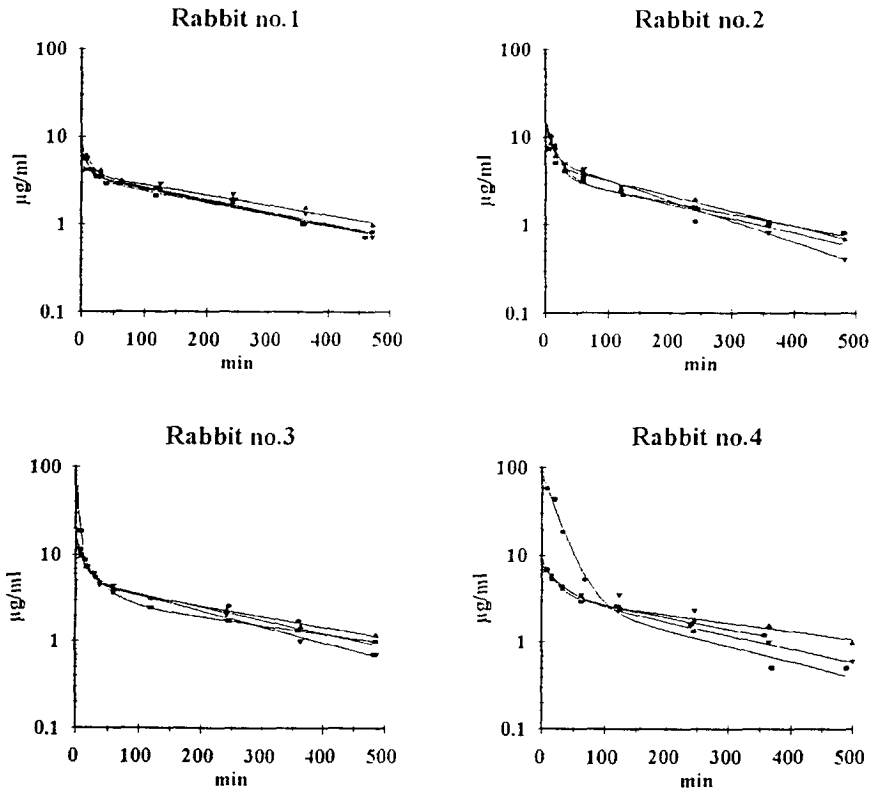

Fig. 3. Plasma concentration–time profiles of tocainide enantiomers after i.v. administration of 25 mg/kg tocainide racemate (▲, *R*-toc; ▼, *S*-toc) or 12.5 mg/kg single enantiomer (■, *R*-toc; ●, *S*-toc). Curves represent the best fit from non-linear regression analysis.

Table 2
Pharmacokinetic parameters of tocainide in the rabbit compared with human data

Parameter	Rabbit[a]		Rabbit[b]		Human[c]	
	R	*S*	*R*	*S*	*R*	*S*
$T_{\frac{1}{2}\alpha}$ (h)	0.19	0.18	0.19	0.18	0.08	0.07
$T_{\frac{1}{2}\beta}$ (h)	3.73	3.22	4.17	2.93	9.24	16.9
Vd_1 (l kg^{-1})	1.40	0.94	1.38	1.27	0.39	0.36
Vd_{SS} (l kg^{-1})	3.06	2.09	2.91	2.57	2.09	1.91
Cl_B (l h^{-1} kg^{-1})	0.59	0.52	0.51	0.65	0.17	0.096

[a] Mean ($n = 4$) for rabbits administered with single tocainide enantiomers.
[b] Mean ($n = 4$) for rabbits administered with racemic tocainide.
[c] From Ref. [3].

mers affect the disposition of one another when administered together as the racemate (Fig. 3).

From Table 2 it can be seen that the elimination of tocainide, expressed as $T_{\frac{1}{2}\beta}$, is more rapid in the rabbit than in humans [3]. This is due to a higher clearance of tocainide in the rabbit since the volumes of distribution of both rabbit and man are in the same order of magnitude.

4. Conclusion

The HPLC method described provides a rapid means for the simultaneous determination of tocainide enantiomers in plasma. The quantitation limit of the method permits application to pharmacokinetic studies.

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