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Enantioselective determination of metoprolol acidic metabolite in plasma and urine using liquid chromatography chiral columns: applications to pharmacokinetics

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Abstract

Enantioselective separations on chiral stationary phases with or without derivatization were developed and compared for the HPLC analysis of (+)-(*R*)- and (−)-(*S*)-metoprolol acidic metabolite in human plasma and urine. The enantiomers were analysed in plasma and urine without derivatization on a Chiralcel OD-R column, and in urine after derivatization using methanol in acidic medium on a Chiralcel OD-H column. The quantitation limits were 17 ng of each enantiomer/ml plasma and 0.5 μg of each enantiomer/ml urine using both methods. The confident limits show that the methods are compatible with pharmacokinetic investigations of the enantioselective metabolism of metoprolol. The methods were employed in a metabolism study of racemic metoprolol administered to a patient phenotyped as an extensive metabolizer of debrisoquine. The enantiomeric ratio (+)-(*R*)/(−)-(*S*)-acid metabolite was 1.1 for plasma and 1.2 for urine. Clearances were 0.41 and 0.25 l/h/kg, respectively, for the (+)-(*R*)- and (−)-(*S*)-enantiomers. The correlation coefficients between the urine concentrations of the acid metabolite enantiomers obtained by the two methods were >0.99. The two methods demonstrated interchangeable application to pharmacokinetics.

Keywords: Enantiomer separation; Circular dichroism; Metoprolol

1. Introduction

Metoprolol {1-isopropylamine-3-[4-(2-methoxyethyl)phenoxy]-2-propanol} is a β_1-adrenoceptor selective antagonist clinically used in the racemic form for the treatment of hypertension and ischaemic heart disease. As in the case of most β-blockers, affinity for the β_1-adrenoceptor is significantly higher for (−)-(*S*)-metoprolol [1,2].

Metoprolol is mainly eliminated by hepatic oxida-

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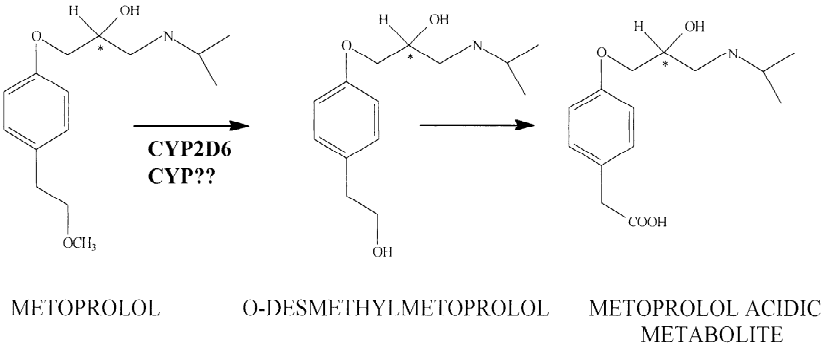

Fig. 1. Oxidative metabolism of metoprolol to the acid metabolite in humans.

tive metabolism. In humans, the main metabolic pathways are *O*-demethylation, oxidative deamination and α-hydroxylation [2] (Fig. 1). The *O*-demethylated metabolite undergoes further oxidation to the corresponding carboxylic acid, relatively the major metabolic pathway, which is responsible for the elimination of 65% of the dose in humans. Polymorphic CYP2D6 participates in metoprolol *O*-demethylation, although other isoforms of CYP are also involved in this process [2].

In vivo and in vitro studies using human liver microsomes have suggested enantioselectivity in the α-hydroxylation and *O*-demethylation pathways [2]. The observed ratio (+)-(*R*)/(−)-(*S*)>1 for the acid metabolite in human plasma and urine indicates that this process has significant enantioselectivity favoring the (+)-(*R*)-metoprolol enantiomer [2,3]. Extensive metabolizers of CYP2D6 have a greater capacity to eliminate the (+)-(*R*)-metoprolol enantiomer and therefore greater clearance of this enantiomer. Consequently, the oxidation phenotype depending on the CYP2D6 activity and enantioselectivity represent the major causes of the inter-individual variability in the pharmacokinetics of this β-blocker [4,5].

Enantioselective direct HPLC analysis of the metoprolol acidic metabolite in urine was described by Kim et al. [6] using a Sumichiral OA-4900 column and fluorescence detection. Although the resolution of the enantiomers was sufficient, the confidence limits of the method for application in pharmacokinetic studies were not reported. The same column was employed by Jung et al. [7] to analyze the enantiomers of the acidic metabolite in a solution prepared from synthesis. Li et al. [8] separated the enantiomers of the acidic metabolite in urine samples after chiral derivatization. In this procedure the carboxyl functional group was blocked by esterification with BF_3 in methanol after solid-phase extraction and then derivatized with the chiral reagent (−)-menthyl chloroformate. The diastereomeric derivatives were analyzed by reversed-phase HPLC. The enantiomers of this metabolite were also directly analyzed in urine by capillary electrophoresis using CM-β-cyclodextrin as chiral selector [9]. The acidic metabolite of metoprolol was analyzed in urine by non-enantioselective HPLC methods simultaneously with metoprolol after solid-phase extraction [10] or dilution and direct injection into the chromatographic system [11,12]. Direct enantioselective HPLC analysis of the metoprolol acidic metabolite in plasma was described by Mistry et al. [3] using a Chirobiotic T column and fluorescence detection after solid-phase extraction.

In the present study we developed and compared procedures for the enantioselective analysis of the metoprolol acidic metabolite in human urine and plasma using direct HPLC and in human urine using HPLC after a derivatization procedure. We also report a preliminary investigation of enantioselectivity in the formation of the metoprolol acidic metabolite after administration of multiple p.o. doses of racemic metoprolol to a hypertensive patient phenotyped as an extensive metabolizer of debrisoquine.

2. Experimental

2.1. Chemicals

(±)-Metoprolol tartrate was obtained from Sigma (St. Louis, MO, USA). (+)-(*R*)-Metoprolol hydrochloride, (−)-(*S*)-metoprolol hydrochloride and the metoprolol metabolites H 117/04 hydrochloride (acidic metabolite), H 119/66 as the *p*-hydroxy benzoate (α-hydroxymetoprolol), H 105/22 as the *p*-hydroxy benzoate (*O*-demethylmetoprolol) and H 104/83 (*N*-dealkylmetoprolol) were kindly donated by Astra Hässle (Mölndal, Sweden). Solvents used as mobile phases were HPLC-grade from Merck (Darmstadt, Germany). Other reagents were of analytical-reagent grade from Merck. Sodium hydroxide solution and sodium chloride were washed twice with diisopropyl ether–dichloromethane (1:1, v/v). Water was purified with a Milli-Q Plus ultra-pure water system (Millipore, Bedford, MA, USA).

2.2. Apparatus

The HPLC system consisted of a Model SIL-10ADVP auto-injector, an LC-10 AD pump for isocratic elution, an RF 551 fluorescence detector operating at 229 nm (λ_{exc}) and 298 nm (λ_{em}) and a C-R6A integrator, all from Shimadzu (Kyoto, Japan). A Model 1595 circular dichroism/UV detector from Jasco (Japan) operating at a wavelength of 274 nm was used to determine the optical rotation of the enantiomers and the UV and CD chromatograms were obtained simultaneously. The chiral columns used were a cellulose tris(dimethylphenyl carbamate) on a 5 μm silica gel substrate for normal phase (Chiralcel OD-H, 150×4.6 mm) with a RP-18 precolumn (Nova-Pak, Waters) and a cellulose tris-(dimethylphenyl carbamate) on a 10 μm silica gel substrate for reversed phase (Chiralcel OD-R, 250×4.6 mm) with a 4×4 mm Licrospher 100 CN precolumn, 10 μm particle size (Merck), both from Daicel Chemical Industries (LO, CA, USA).

2.3. Standard solutions

A standard solution of the (±)-metoprolol acidic metabolite was prepared in methanol at 0.44 mg free base/ml. The diluted solutions were prepared at concentrations of 2.2, 4.4, 8.8, 11, 22, 44, 88 and 110 μg/ml methanol and stored at −20 °C in the dark.

Solutions of the individual metoprolol enantiomers as well as the other metoprolol metabolites (α-hydroxymetoprolol, *O*-demethylmetoprolol and *N*-dealkylmetoprolol) were prepared at a concentration of 0.1 mg/ml free base in methanol. All of the standard solutions were stored at −20 °C in the dark.

2.4. Sample preparation

2.4.1. Sample clean-up

Plasma samples (400 μl) were alkalinized with 25 μl of a 1 *M* sodium hydroxide solution and the proteins were precipitated with 3.0 ml acetonitrile. After shaking in a vortex for 30 s the mixtures were centrifuged at 2000 *g* for 5 min and 3 ml of the upper phases were transferred to conical tubes containing the same volume of dichloromethane–diisopropyl ether (1:1, v/v). The mixtures were shaken in a vortex for an additional 1 min, centrifuged at 2500 *g* for 5 min and the aqueous phases (100 μl) were transferred to auto-injector tubes. After sample acidification with 15 μl of 0.225 *M* perchloric acid, 50 μl of this mixture was injected into the chromatographic system.

Urine samples (25 μl) were alkalinized with 200 μl of a 0.02 *M* disodium tetraborate aqueous solution. After shaking in a vortex for 30 s, 3.0 ml dichloromethane–diisopropyl ether (1:1, v/v) was added. The samples were shaken for 1 min in a vortex and centrifuged at 2000 *g* for 5 min. The aqueous phases (100 μl) were transferred to auto-injector tubes, 10 μl of a 0.4 *M* perchloric acid solution was added to acidify the samples and 20 μl of the mixture was injected into the chromatographic system.

2.4.2. Solid-phase extraction (SPE)–derivatization

Urine samples (25 μl) were supplemented with 200 μl of a 0.02 *M* aqueous solution of disodium tetraborate. The mixtures were applied to a Supelclean LC-18 SPE column (3.0 ml tubes, Supelco, Bellefonte, PA, USA) preconditioned by sequential washing with methanol (2.0 ml) and a 0.02 *M* aqueous solution of disodium tetraborate (2.0 ml). Cartridges were washed with 2.0 ml water. Excess

water was removed by leaving the cartridges in a vacuum system for 20 min, washing with 1.0 ml dichloromethane and standing in the vacuum system for an additional 4 min. The analytes were eluted from the cartridges with 4.0 ml methanol. The eluates were evaporated to dryness under a flow of air at room temperature. The residues obtained in the SPE procedure were dissolved in 2 ml of 0.5 *M* hydrochloric acid in anhydrous methanol solution and 200 mg anhydrous sodium sulfate was added. The mixtures were shaken in a vortex for 30 s and the derivatization reaction was performed by heating the mixtures to 60 °C for 30 min. After the addition of 4 ml 0.1 *M* phosphate buffer, pH 7.0, the derivatives were extracted with 7.0 ml chloroform for 3 min in a vortex shaker. The samples were centrifuged at 1800 *g* for 5 min and the organic phases (6.5 ml) were transferred to conic tubes and evaporated to dryness under a flow of air at room temperature. The residues obtained were dissolved in 50 μl of the mobile phase and 20 μl was injected into the chromatographic system.

2.5. Chromatography

2.5.1. Chiralcel OD-R separation

Separation of the acid metabolite enantiomers was performed on a Chiralcel OD-R column with a mixture of 0.5 *M* sodium perchlorate/perchloric acid (pH 3.0)–acetonitrile (85:15, v/v) as mobile phase. The flow-rate was 0.4 ml/min at 25 °C.

2.5.2. Chiralcel OD-H separation

Separation of the derivative enantiomers was performed on a Chiralcel OD-H column with the mobile phase consisting of a mixture of hexane–ethanol–diethylamine (90:10:0.2, v/v). The flow-rate was 1.0 ml/min at 25 °C.

2.5.3. Identification of the enantiomers

The residue obtained in the SPE derivatization procedure of a highly concentrated sample (60 μg/ml) was injected into the HPLC system described in Section 2.5.2 using a circular dichroism detector to determine the optical rotation of each enantiomer. The results were compared with the CD spectra of pure metoprolol enantiomers.

2.6. Calibration curves and validation of the methods

The human plasma and urine (blank samples) employed for the validation of the analytical methods were initially used to determine the absence of interference peaks. The calibration curves were constructed from 25 μl samples of blank urine or 400 μl samples of blank plasma spiked with known amounts of acid metabolite. The linear regression equations and the correlation coefficients were obtained from the heights of the peaks plotted against their respective concentrations (0.5–110.0 μg/ml urine and 41–1650 ng/ml plasma for each enantiomer).

The linearity of each method was studied by the analysis of plasma and urine samples spiked with increasing metoprolol acidic metabolite concentrations in relation to those employed for the construction of the calibration curves. The quantitation limit was determined as the lowest intra-assay concentration ($n=5$) analyzed with a coefficient of variation of less than 15%.

The recovery of acid metabolite enantiomers was evaluated only in the SPE–derivatization method by comparing the heights of the peaks obtained after the solid-phase extraction and derivatization procedures with the heights of the peaks obtained after derivatization only.

The precision of each method was assessed by determining the intra- ($n=8$) and inter-assay ($n=5$) coefficients of variation of the analysis of spiked blank plasma and urine samples.

The interference of metoprolol, the other metoprolol metabolites and other drugs possibly co-administered with the antihypertensive agent was evaluated by the analysis of blank samples spiked with drug concentrations similar to those observed at therapeutic doses.

2.7. Clinical sampling

The methods were applied to the investigation of enantioselectivity in the formation of the metoprolol acidic metabolite after racemic metoprolol administration in a multiple p.o. dose (200 mg/24 h/7 days). The hypertensive patient (a 53-year-old man, white; urinary ratio debrisoquine/4-hydroxydebrisoquine

0.53) was included in the study after giving written consent to participate. The presence of mild chronic renal failure was observed by a creatinine clearance of 51.5 ml/min. After a 12 h fast, the patient received two 100 mg metoprolol tartrate tablets (Seloken, Astra, Brazil) with interruption of the subsequent dose. Serial blood samples were collected 0–24 h after metoprolol administration. Urine was collected in five fractions over a period of 36 h after drug administration. The total volume of urine in each fraction was measured, an aliquot removed and stored at −20 °C.

The pharmacokinetic parameters obtained using the clean-up procedures were calculated within one time dosing interval by fitting the data to a mono-compartmental open model as described elsewhere [13].

3. Results and discussion

In the present study we developed and compared two methods for the analysis of the enantiomers of the acidic metabolite of metoprolol in human urine and one method in plasma. The method using the clean-up procedure was carried out on a Chiralcel OD-R chiral column (reversed phase), whereas the method using solid-phase extraction followed by esterification of the carboxylic group was carried out on a Chiralcel OD-H chiral column (normal phase). Derivatization blocked the acid function, permitting this polar metabolite to be assayed by normal-phase HPLC.

Analysis of the blank plasma and urine samples collected from different healthy volunteers did not show interference of the endogenous components with the acid metabolite enantiomers eluted directly from the Chiralcel OD-R column (Fig. 2I and III) or the derivatives eluted from the Chiralcel OD-H column (Fig. 2II).

Since the acidic metabolite has both amino and carboxylic acid functions, it is difficult to extract from aqueous media using a liquid–liquid procedure. Study of the recovery (Table 1) revealed values greater than 90% for both metoprolol acidic metabolite enantiomers extracted from urine by the solid-phase procedure. Previously reported methods that analyzed the metoprolol acidic metabolite in plasma and urine employed procedures of solid-phase extraction (C-18 columns) [3,8,10] or direct injection of the samples after dilution with the mobile phase [11] or water [12]. Metoprolol and its other metabolites were efficiently removed from plasma and urine by liquid–liquid clean-up, avoiding interference, and we observed, by injecting the dried organic phase, that the acidic metabolite was not lost during this procedure.

The peak height versus concentration curves for both acid metabolite enantiomers were constructed in the 0.5–220 μg/ml urine and 17–3300 ng/ml plasma range, with correlation coefficients greater than 0.99 for the two methods (Table 1). The quantitation limits determined by the analysis of plasma samples (400 μl) were 17 ng/ml, and for urine samples (25 μl) they were 0.5 μg/ml for both enantiomers analyzed on Chiralcel OD-R and Chiralcel OD-H columns (Table 1). These data show that the method developed to analyze the enantiomers of the acid metabolite in plasma samples is as sensitive as the method described by Mistry et al. [3] and both methods developed to analyze the enantiomers of the acid metabolite in urine samples are almost 20 times more sensitive than the HPLC method described by Li et al. [8] and two times more sensitive than the capillary electrophoresis method described by Lim et al. [9].

The coefficients of variation obtained in the study of within-day and between-day precision were less than 15%, ensuring the repeatability of the results in the 17–3300 ng/ml plasma and 0.5–110 μg/ml urine range. The data obtained in the precision study also demonstrated that an internal standard is not required in the analysis.

Analysis of the selectivity (Table 2) permitted the application of the methods to the study of the kinetic disposition in hypertensive patients submitted to therapy with racemic metoprolol in combination with other drugs. Drugs such as acetaminophen, captopril, cimetidine, carbamazepine, diazepam, hydrochlorothiazide, imipramine, furosemide, ranitidine, quinidine, diclofenac, digoxin and verapamil were not detected in the chromatographic system. Metoprolol and the other metoprolol metabolites were efficiently removed by the clean-up procedure and did not interfere with the Chiralcel OD-H system.

The elution order was determined using a circular

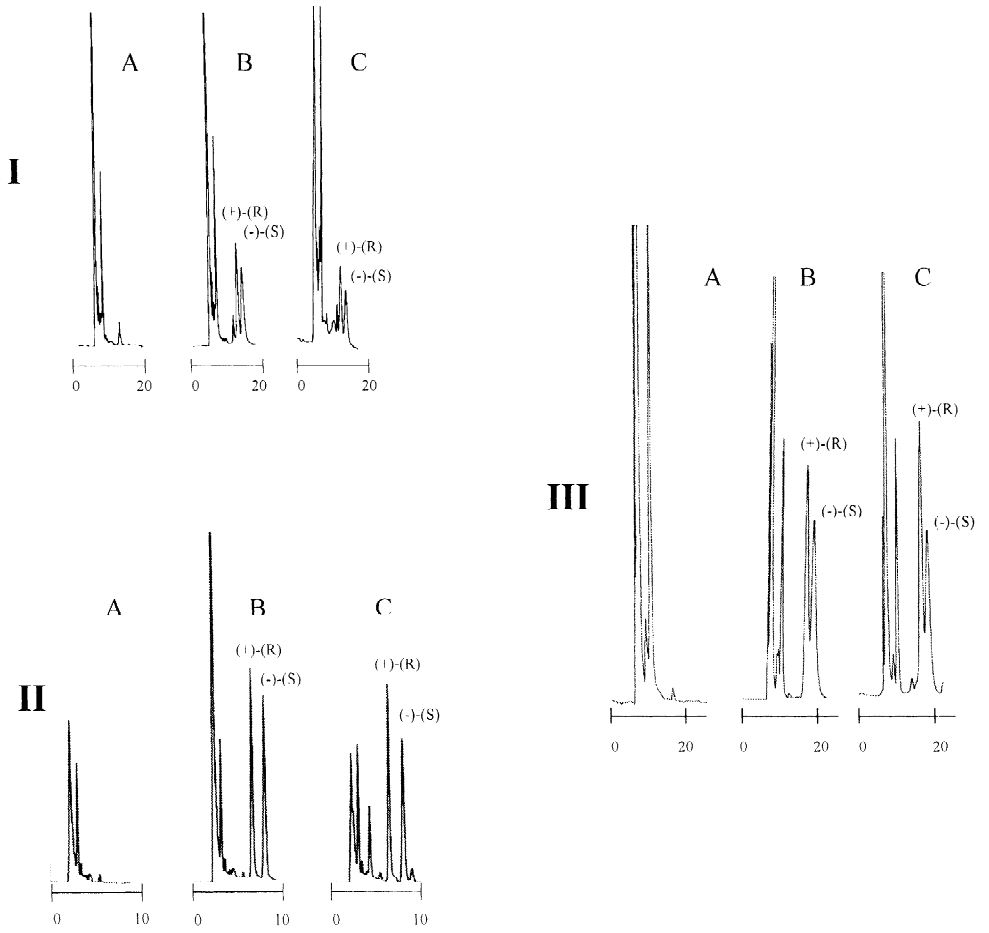

Fig. 2. (I) Urine clean-up—Chiralcel OD-R. (II) Urine SPE–derivatization—Chiralcel OD-H. (III) Plasma clean-up—Chiralcel OD-R. Chromatograms of (A) the blank, (B) the blank spiked with acid metabolite enantiomers (687 ng/ml plasma; 22 μg/ml urine) and (C) a sample from a patient treated with racemic metoprolol.

dichroism detector to identify the two resolved enantiomers without and after the derivatization procedure. As can be observed in Fig. 3, the first enantiomer eluted from the Chiralcel OD-H column has a positive sign and the second enantiomer has a negative sign in the 274 nm CD chromatogram. The spectra of the pure metoprolol enantiomers present a positive sign at 274 nm for (+)-(*R*)-metoprolol and a negative sign at the same wavelength for (−)-(*S*)-metoprolol. On the basis of the slight difference in metabolite structure without any change in the chiral center, we can assign the same absolute configuration to the metoprolol acidic metabolite enantiomers, determining the elution order as (+)-(*R*)-acid metabolite (5.23 min) and (−)-(*S*)-acid metabolite (6.78 min).

The methods developed and validated were employed in an investigation of enantioselectivity in the formation of the metoprolol acidic metabolite after administration of racemic metoprolol p.o. in a multiple-dose regimen of the tartrate salt (200 mg/24 h) to one patient phenotyped as an extensive metabolizer of debrisoquine (metabolic ratio debrisoquine/4-hydroxydebrisoquine 0.53) [14,15]. The correlation coefficients obtained between the urine concentrations of the enantiomers of the acidic metabo-

Table 1
Confidence limits of the methods for the analysis of metoprolol acidic metabolite

Acid metabolite	Recovery (%)	Quantitation limit (C.V.[a], %)	Linearity (r^b)
Sample clean-up			
Plasma (ng/ml)			
(+)-(*R*)-	–	17 (6.81)	17–3300 (0.995)
(−)-(*S*)-	–	17 (12.3)	17–3300 (0.998)
Urine (μg/ml)			
(+)-(*R*)-	–	0.5 (9.4)	0.5–220 (0.993)
(−)-(*S*)-	–	0.5 (5.5)	0.5–220 (0.993)
SPE derivatization			
Urine			
(+)-(*R*)-	90.7	0.5 (4.1)	0.5–110 (0.989)
(−)-(*S*)-	92.4	0.5 (7.9)	0.5–110 (0.993)

[a] C.V., coefficient of variation.
[b] *r*, correlation coefficient.

lite of metoprolol determined by the methods with and without derivatization were greater than 0.995 and permitted the quantitation of both enantiomers up to 36 h after metoprolol administration.

The plasma concentration versus time curves (Fig. 4 and Table 3) demonstrated greater elimination half-lives for the (+)-(*R*)-acid metabolite and a ratio of the area under the plasma concentration versus time curve ($AUC^{0-24}_{(+)\text{-}(R)}/AUC^{0-24}_{(-)\text{-}(S)}$) of 1.12 for the patient investigated. The urine excretion rate versus midpoint time curves (Fig. 4 and Table 3) demonstrated a ratio of the total amount excreted, $Ae_{(+)\text{-}(R)}/Ae_{(-)\text{-}(S)}$ of 1.22. The greater concentration of the (+)-(*R*)-metoprolol acidic metabolite in plasma and urine suggests preferable formation of this enantiomer. These results support the thesis that this metabolic pathway is at least partly responsible for the accumulation of (−)-(*S*)-metoprolol in humans [2].

4. Conclusion

The method using sample clean-up without derivatization and the method using solid-phase extraction followed by derivatization presented a sensitivity, selectivity and precision suitable for application in studies of the enantioselective metabolism of metoprolol administered in a p.o. multiple-dose

Table 2
Study of the selectivity of the methods for the analysis of metoprolol acidic metabolite enantiomers in plasma and urine

Drug	Conc. (μg/ml)	Retention time (min)	
		Sample clean-up	SPE–derivatization
(+)-(*R*)-Acid metabolite	11.0	16.6	5.4
(−)-(*S*)-Acid metabolite	11.0	18.6	6.9
Metoprolol	0.1	43.1/51.8[a]	3.3/4.3
N-Dealkylmetoprolol	0.1	21.6[a]	12.1/13.8
O-Demethylmetoprolol	0.1	13.6/15.3[a]	ND
α-Hydroxymetoprolol	0.1	11.3/12.2[a]	4.7

ND, not detected (0 to 45 min).
[a] Removed by the clean-up procedure.

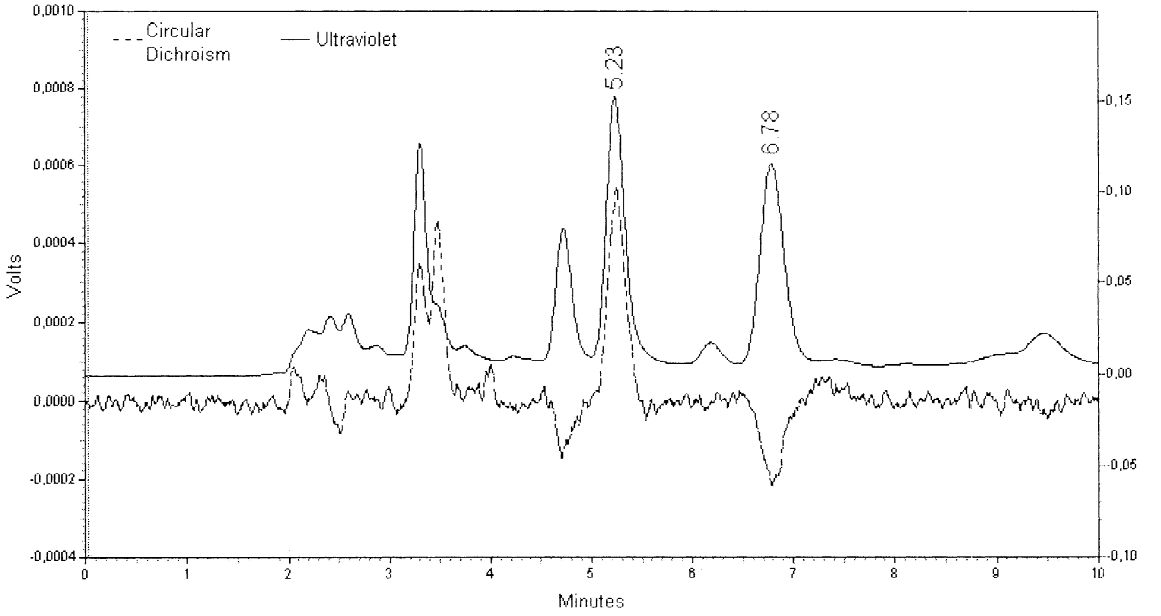

Fig. 3. Racemic metoprolol acidic metabolite resolved on a Chiralcel OD-H column after SPE–derivatization and detected using CD/UV detection. (+)-(*R*)-Acidic metabolite (5.23 min; positive peak at CD) and (−)-(*S*)-acidic metabolite (6.78 min; negative peak at CD).

regimen. The method using the sample clean-up procedure (Chiralcel OD-R) is faster and it is recommended when the clinical study involves a large number of urine samples.

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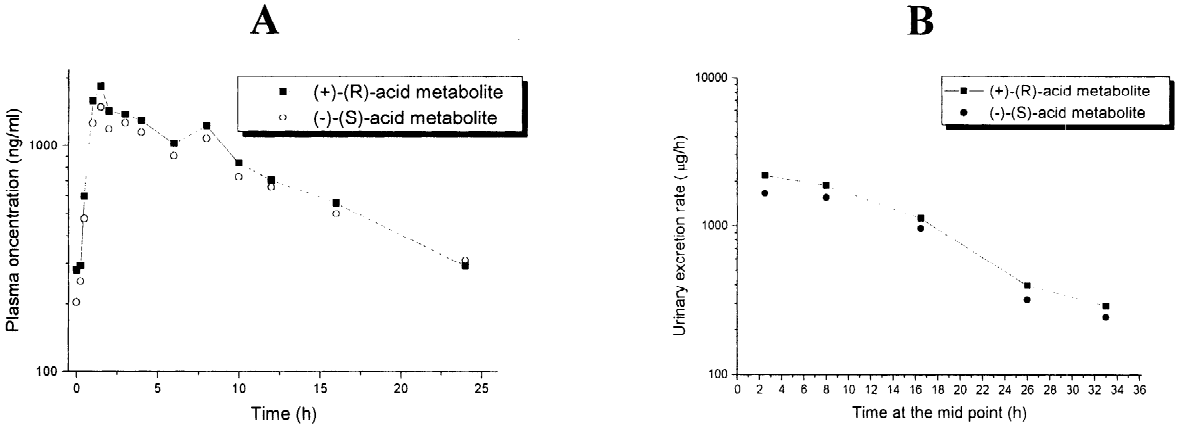

Fig. 4. Plasma concentration versus time curves (A) and urinary excretion rate versus time at the midpoint (B) for the metoprolol acidic metabolite enantiomers in a patient treated with a 200 mg/24 h dose of racemic metoprolol.

Table 3
Pharmacokinetic parameters of metoprolol acidic metabolite enantiomers

Parameter	(+)-(*R*)-Acid metabolite		(−)-(*S*)-Acid metabolite
C_{max} (μg/ml)	1.84		1.48
t_{max} (h)	1.5		1.5
$t_{1/2}$ β (h)	9.0		8.4
AUC^{0-24} (μg h/ml)	19.26		17.18
$AUC^{0-24}_{(+)/(-)}$		1.12	
Ae^{0-24} (mg)	39.41		32.18
F_{el}/f (%)	25.23		20.60
K_{el} (h^{-1})	0.0787		0.0801
Cl_M (l/h/kg)	0.4073		0.2509
$Ae^{0-24}_{(+)/(-)}$		1.22	

C_{max}, maximum plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2}$ β, half-life; AUC^{0-24}, area under the plasma concentration–time curve in dose interval; Ae^{0-24}, total amount excreted in urine in dose interval; F_{el}/f, fraction of dose excreted as metoprolol acidic metabolite; K_{el}, elimination rate constant; Cl_M, clearance.

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