CHIRALITY 15:542–549 (2003)

Stereoselective Metabolism of Metoprolol: Enantioselectivity of α-Hydroxymetoprolol in Plasma and Urine

PAULA MACEDO CERQUEIRA,[1] EVANDRO JOSÉ CESARINO,[2] CARLO BERTUCCI,[3] PIERINA SUELI BONATO,[2] AND VERA LUCIA LANCHOTE[2]*

[1]*Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brazil*
[2]*Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil*
[3]*Dipartimento di Scienze Farmaceutiche, Università di Bologna, Bologna, Italy*

ABSTRACT Direct stereoselective separation on chiral stationary phase was developed for HPLC analysis of the four stereoisomers of α-hydroxymetoprolol in human plasma and urine. Plasma samples were prepared using solid-phase extraction columns and urine samples were prepared by liquid–liquid extraction. The stereoisomers were separated on a Chiralpak® AD column at 24°C with fluorescence detection and a mobile phase consisting of a mixture of hexane:ethanol:isopropanol:diethylamine (88:10.2:1.8:0.2) for plasma samples and hexane:ethanol:diethylamine (88:12:0.2) for urine samples. Calibration curves for the individual stereoisomers were linear within the concentration range of 2.0–200 ng/ml plasma or 0.125–25 μg/ml urine. The methods were validated with intra- and interday variations less than 15%. The absolute configuration of the pure stereoisomers were assigned by circular dichroism spectra. The methods were employed to determine the concentrations of α-hydroxymetoprolol stereoisomers in a metabolism study of multiple-dose administration of racemic metoprolol to hypertensive patients phenotyped as extensive metabolizers of debrisoquine. We observed stereoselectivity in the α-hydroxymetoprolol formation favoring the new 1′*R* chiral center from both metoprolol enantiomers ($AUC^{0-24}_{1'R/1'S}$ = 3.02). The similar renal clearances (Cl_R) of the four stereoisomers demonstrated absence of stereoselectivity in their renal excretion. (–)-(*S*)-metoprolol was slightly more α-hydroxylated than its antipode ($AUC^{0-24}_{2S/2R}$ = 1.19), suggesting that this pathway is not responsible for plasma accumulation of this enantiomer in humans. *Chirality 15:542–549, 2003.*

KEY WORDS: α-hydroxymetoprolol; metoprolol; stereoisomers; metabolism; enantioselective HPLC; CYP2D6

Metoprolol is a β_1-adrenoceptor selective antagonist clinically used as the racemic form for the treatment of hypertension and ischemic heart disease.[1] It is mainly eliminated by hepatic oxidative metabolism. About 85% of an administered dose is excreted in the urine as metabolites, with a small amount of the unchanged parent compound.[2] Stereoselectivity in metoprolol pharmacokinetics has been reported with higher plasma concentrations of (–)-(*S*)-metoprolol and higher renal excretion of (+)-(*R*)-metoprolol in healthy volunteers receiving a single oral dose of racemic metoprolol[3,4,5] or at steady-state[6,7] and in hypertensive patients at steady state.[8]

In humans, the main metabolic pathways of metoprolol are α-hydroxylation, O-demethylation (Fig. 1), and oxidative deamination. The α-hydroxylation pathway contributes to the elimination of only 10% of the dose but it results in the formation of the pharmacologically active α-hydroxymetoprolol, with 1/10 of the potency of metoprolol in reducing exercise heart rate.[2,9] This has been suggested to be a major contributor to the effects of metoprolol in reduction of essential tremor.[10]

The formation of α-hydroxymetoprolol has the potential to yield four isomeric products because a new chiral center is formed upon benzylic hydroxylation.[11] This pathway is catalyzed by CYP2D6, which is polymorphic and cosegregates with the metabolism of debrisoquine.[12] In vivo and in vitro studies using human liver microsomes have suggested stereoselectivity of the α-hydroxylation process, favoring formation of a new 1′*R* chiral center from both

*Correspondence to: Vera Lucia Lanchote, Faculdade de Ciências Farmacêuticas de Ribeirão Preto-USP, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Avenida do Café s/n. Campus da USP, 14040-903, Ribeirão Preto, SP, Brazil. E-mail: lanchote@fcfrp.usp.br
Received for publication 5 September 2002; Accepted 6 February 2003

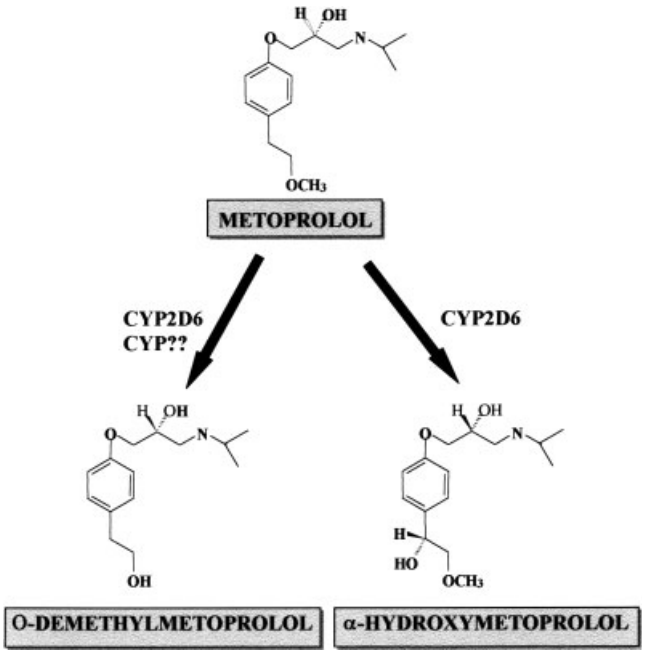

Fig. 1. Major pathways of metoprolol metabolism in man.

enantiomers of metoprolol.[9,11] The formation of α-hydroxymetoprolol is impaired in poor metabolizers of debrisoquine.[13] Consequently, the CYP2D6 oxidation phenotype and stereoselectivity represent the major factors that yield interindividual variability in the metabolism of metoprolol.[4,7,9]

Metoprolol enantiomers have been separated by direct analysis using four types of chromatographic columns based on chiral stationary phases: α_1-acid glycoprotein,[14–17] cellulose tris(3,5-dimethylphenylcarbamate),[18–26] amylose tris(3,5-dimethylphenylcarbamate),[8,24] and the antibiotic Teicoplanin.[27] Schuster et al.[28] used 2,3,4,5-tetra-O-acetyl-β-d-glucopyranosyl isothiocianate (GITC) as derivatization reagent to assay metoprolol enantiomers and α-hydroxymetoprolol diastereomers indirectly. Only Balmér et al.[21] developed a method able to separate the four stereoisomers of α-hydroxymetoprolol. This separation was performed on a Chiralcel OD (cellulose tris(3,5-dimethylphenylcarbamate coated on silica-gel) column at 25°C, 10% 1-propanol, 0.1% diethylamine, and 600 mg/l water in hexane as eluent at a flow rate of 0.5 ml/min; and it takes more than 50 min to elute all the stereoisomers. The absolute configuration of the four α-hydroxymetoprolol stereoisomers was not determined in this work, with the peaks being identified only by the metoprolol chiral center after administration of the (–)-(*S*)-metoprolol in a dog. Quite recently, the same column has been successfully applied by Kim et al.[26] and the elution order was assigned on the basis of the metabolic process of the single enantiomers of metoprolol. An HPLC assay of α-hydroxymetoprolol was reported also by Mistry et al.,[27] but not all the stereoisomers were resolved.

Murthy et al.[9] studied the α-hydroxylation process analyzing the urinary α-hydroxymetoprolol stereoisomers in eight extensive metabolizers of debrisoquine and they observed preferential formation of the new 1′*R* chiral center over a new 1′*S* chiral center by 3-fold, regardless of the chirality of the propanolaminic sidechain. The assay was performed using HPLC followed by GC-MS after administration of a single dose of pseudoracemic metoprolol prepared by dissolving equimolar amounts of tartrate salts of (2*S*)-metoprolol-d_2 and (2*R*)-metoprolol-d_0 in water. There are no studies in human plasma to confirm that the stereoselectivity observed in the urinary α-hydroxymetoprolol results from the preferential formation of the 1′*R* stereoisomers or if it is a consequence of stereoselective renal excretion.

In the present study, we describe a procedure for the stereoselective analysis of α-hydroxymetoprolol in human plasma and urine using direct enantioselective HPLC and we determined the absolute configuration of each peak using circular dichroism. We also report an investigation of stereoselectivity in the metabolism of metoprolol administered in multiple doses to hypertensive Caucasian patients phenotyped as extensive metabolizers of debrisoquine.[29]

EXPERIMENTAL

Chemicals

(±)-Metoprolol tartrate was obtained from Sigma (St. Louis, MO) and the metoprolol metabolites H 119/66 as p-OH-benzoate (α-hydroxymetoprolol), H 117/04 hydrochloride (acid metabolite), H 105/22 as p-OH benzoate (O-demethylmetoprolol), and H 104/83 were kindly donated by Astra Hässle AB, Sweden. The (1′*S*/1′*R*:70/30)-α-hydroxymetoprolol sample was kindly donated by Prof. Wendel L. Nelson. Solvents used as mobile phases were HPLC-grade from Merck (Darmstadt, Germany). Other reagents were of analytical-reagent grade. Water was purified with a Milli-Q Plus ultrapure water system (Millipore, Bedford, MA).

Apparatus

The HPLC system consisted of a model 7125 manual loop injector (Rheodyne, Berkeley, CA), a pump for isocratic elution, a model RF 551 fluorescence detector operating at 229 nm (λ_{exc}) and 298 nm (λ_{em}), and a model C-R6A integrator (Shimadzu, Kyoto, Japan). The chiral column used was an amylose tris(3,5-dimethylphenylcarbamate) coated on 10 μm silica-gel (Chiralpak® AD, 250 × 4.6 mm) from Daicel Chemical Industries (Tokyo, Japan) with a 4 × 4 mm Lichrospher® 100 CN precolumn, 10 μm particle size (Merck). The flow rate was 1.2 ml/min at 24°C.

The circular dichroism spectra were obtained with a Jobin Yvon CD6 dichrograph (Jobin Yvon, Stanmore, England) and a Jasco 810 spectropolarimeter (Jasco, Japan). Both instruments were interfaced to personal computers to acquire and elaborate data. All measurements were carried out at room temperature using 0.5 and 1 mm pathlength cells.

Standard Solutions

Standard solutions of (±)-α-hydroxymetoprolol as the p-hydroxybenzoate were prepared in methanol at 0.2 mg free base/ml. The diluted solutions were prepared at concen-

trations of 0.8, 1.6, 2.0, 4.0, 8.0, 10, 20, 40, and 100 µg/ml methanol for analysis of plasma and urine samples. A solution of (±)-α-hydroxymetoprolol at 20 mg free base was prepared in 1 ml ethanol for purification of the four stereoisomers. Solutions of the metoprolol as well as the other metoprolol metabolites were prepared at concentrations of 0.1 mg/ml free base in methanol. All of the standard solutions were stored at –20°C in the dark.

Sodium chloride (Merck) and sodium hydroxide solutions were washed twice with di-isopropyl ether:dichloromethane 1:1, v/v.

Sample Preparation

Urine samples. Urine samples (100 µl) were alkalinized with 25 µl of a 1 M NaOH aqueous solution. After mixing for 30 sec and a 5-min rest, 10 mg sodium chloride and 2.0 ml dichloromethane:di-isopropyl ether (1:1, v/v) were added. The samples were shaken for 30 min in a horizontal shaker (220 ± 10 cycles/min) and centrifuged at 2,000*g* for 5 min and the organic phases were transferred to conical tubes and evaporated to dryness under an airflow at room temperature. The residues obtained were dissolved in 200 µl of the mobile phase consisting of a mixture of hexane:ethanol:diethylamine (88:12:0.2, v/v/v). The samples were injected with a 50-µl injection loop.

Plasma samples. Plasma samples (1,000 µl) were supplemented with 500 µl of a 0.02 M disodium tetraborate aqueous solution. The mixtures were applied to LC-18 SPE columns (Supelclean® LC-18 SPE, 3.0 ml tubes; Supelco, Bellefonte, PA) preconditioned by sequential washing with methanol (2.0 ml) and a 0.02 M disodium tetraborate solution (2.0 ml). Cartridges were washed successively with 2.0 ml 0.02 M disodium tetraborate solution and 2.0 ml water. Excess water was removed by leaving the cartridges in a vacuum system for 20 min, washing with 1.0 ml acetonitrile, and maintenance in a vacuum system for an additional 4 min. The analytes were eluted from the cartridges with 6.0 ml methanol. The eluates were evaporated to dryness under an airflow at room temperature. The residues obtained were dissolved in 100 µl of the mobile phase consisting of a mixture of hexane:ethanol:isopropanol: diethylamine (88:10.2:1.8:0.2, v/v/v/v) and 50 µl were injected into the chromatograph.

Calibration Curves and Validation of the Method

The pooled human plasma and urine employed for the validation of the analytical method were initially used to determine the absence of interference peaks with retention times equal or close to those of α-hydroxymetoprolol stereoisomers.

The calibration curves were constructed from 1,000-µl samples of blank plasma or 100-µl urine samples spiked with diluted standard solution of α-hydroxymetoprolol. The linear regression equations and the correlation coefficients were obtained from the heights of the peaks plotted against their respective plasma concentrations (5–50 ng/ml plasma or 0.125–25 µg/ml urine for each α-hydroxymetoprolol isomer).

The recovery of α-hydroxymetoprolol stereoisomers was evaluated by comparing the heights of the peaks obtained after extraction with the heights of the peaks obtained after direct injection of the standard solutions.

The quantitation limit was determined as the lowest intra-assay concentration (n = 5) analyzed with a coefficient of variation of less than 20%.

The linearity of each method was studied by analysis of samples spiked with increasing α-hydroxymetoprolol concentrations in relation to those employed for the construction of the calibration curve. The method was considered to be linear up to the highest concentration studied, having a linear relationship with the detector response.

The precision of each method was evaluated by analysis of blank plasma or urine samples spiked with two different α-hydroxymetoprolol concentrations and the results were reported as within-day (n = 8) and between-day (n = 5) coefficients of variation.

The interference of metoprolol, the other metabolites, and other drugs possibly coadministered with the antihypertensive agent in the α-hydroxymetoprolol stereoisomers assay was evaluated by analysis of blank plasma

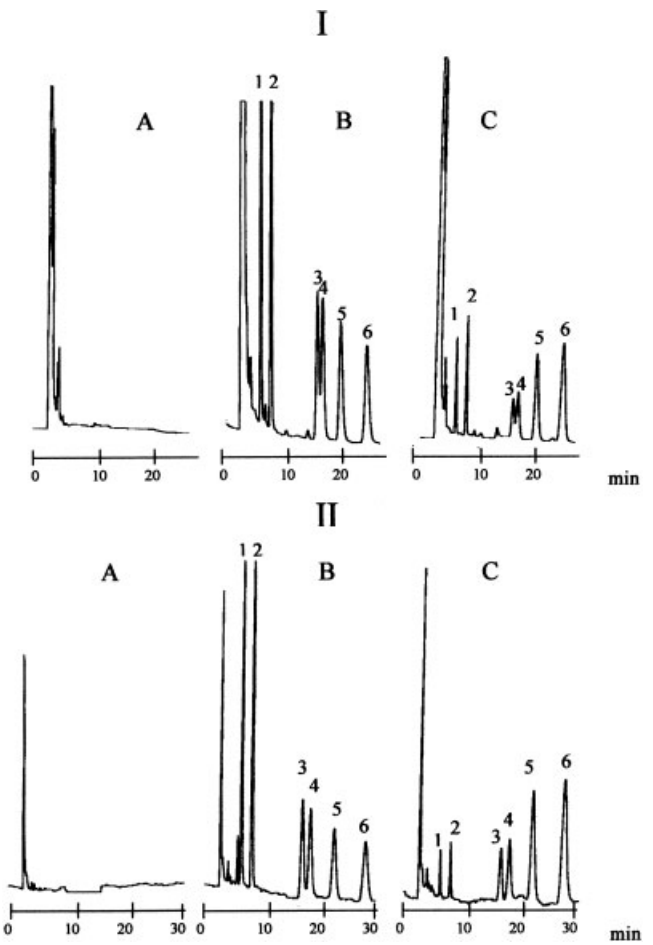

Fig. 2. I: *Plasma analysis;* **II:** *Urine analysis.* Chromatograms of **(A)** blank samples; **(B)** blank samples spiked with *rac*-metoprolol and *rac*-α-hydroxymetoprolol, and **(C)** samples from a patient treated with *rac*-metoprolol tartrate. (1) (+)-(*R*)-metoprolol; (2) (–)-(*S*)-metoprolol; (3) (1′*S*,2*R*)-α-hydroxymetoprolol; (4) (1′*S*,2*S*)-α-hydroxymetoprolol; (5) (1′*R*,2*R*)-α-hydroxymetoprolol; (6) (1′*R*,2*S*)-α-hydroxymetoprolol.

TABLE 1. Demographic, clinical, and laboratory data of the patients

Patient (sex)	Age (years)	Weight (kg)	Height (cm)	Cl_{CR} ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$)	Coadministered drugs
1 (M)	32	60	160	110.8	—
2 (M)	45	78	174	141.6	—
3 (F)	76	67	166	56.9	—
4 (F)	75	49	155	69.0	—
5 (F)	63	67	169	131.2	Estradiol benzoate
6 (M)	55	103	168	90.3	Acetylsalicylic acid, propatyl nitrate
7 (F)	43	72	147	104.6	—
8 (M)	49	84	170	101.0	Bromazepam
9 (F)	28	76	169	65.6	N-Butylscopolamine
10 (F)	61	91	160	—	—

CR = creatinine; — not determined.

spiked with drug concentrations similar to those observed at therapeutic doses.

Purification and Determination of the Absolute Configuration

The 20 mg/ml solution of (±)-α-hydroxymetoprolol was injected successively into the HPLC system using a 20-µl loop. The pure stereoisomers were collected and in the end an aliquot of each solution was injected to assure that the stereoisomers were at least 90% pure. The mobile phase was evaporated to dryness under an airflow at room temperature, resulting in a 4.4 mg of peak 3 (RT = 16.5 min), 5.4 mg of peak 4 (RT = 17.9 min), 5.3 mg of peak 5 (RT = 22.2 min), and 5.5 mg of peak 6 (RT = 27 min) (Fig. 2II). The residues obtained were dissolved in methanol (1 ml) and transferred to a circular dichroism (CD) cell of 0.5 mm optical length (200 µl volume). The measurements were taken from 210 nm to 320 nm at each 0.25 nm with 1 sec for integration. The procedure was repeated three times and the average spectrum was subtracted from blank spectrum (methanol).

Clinical Sampling

The direct methods to analyze plasmatic and urinary α-hydroxymetoprolol stereoisomers were applied for the investigation of stereoselectivity in the metabolism of metoprolol administered in the racemic form in a multiple p.o. dose. The study protocol was approved by the Research Ethics Committee of the local hospital (Ribeirão Preto, SP, Brazil). Patients were included in the study after giving written consent to participate and after hepatic, renal, and cardiac functions were found to be normal by clinical examination and on the basis of laboratory data (Table 1). The patients were treated with racemic metoprolol tartrate (Seloken®, Astra, Brazil) in a multiple dose schedule (2 tablets of 100 mg each every 24 h) for 7 days. On the seventh day of treatment the patients were admitted to Hospital São Paulo, Ribeirão Preto, SP, Brazil, and received the daily dose of metoprolol with 200 ml of water under fasting conditions. Blood samples were removed via an intravenous catheter at zero, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 20, 22, and 24 h after metoprolol administration, with heparin (Liquemine® 5000 IU, Roche, Nutley, NJ) used as anticoagulant. The blood samples were centrifuged at 1,800*g* for 10 min and the plasma stored at −20°C until chromatographic analysis. Urine was collected at each 6-h period until 24 h after drug administration.

The pharmacokinetics of α-hydroxymetoprolol were analyzed on the basis of the open monocompartmental model.[30] The elimination half-life ($t_{1/2}\beta$) was determined

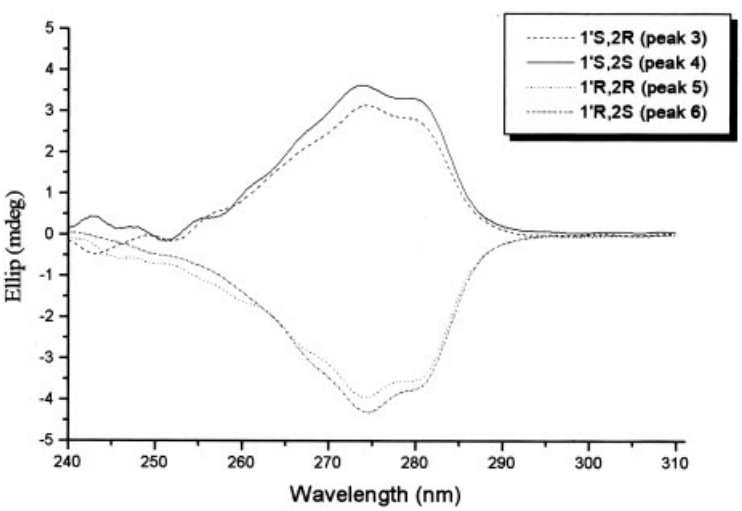

Fig. 3. Circular dichroism spectra of isolated α-hydroxymetoprolol stereoisomers.

TABLE 2. Confidence limits of the methods for the analysis of α-hydroxymetoprolol stereoisomers in plasma and urine samples

	Recovery (%)	Quantitation limit (ng/ml)	Linearity (ng/ml)
Plasma			
(1′*S*,2*R*)	93.3	2.0	2–200
(1′*S*,2*S*)	92.0	2.0	2–200
(1′*R*,2*R*)	101.0	2.0	2–200
(1′*R*,2*S*)	88.5	2.0	2–200
Urine			
(1′*S*,2*R*)	66.3	125.0	125–25000
(1′*S*,2*S*)	64.8	125.0	125–25000
(1′*R*,2*R*)	62.3	125.0	125–25000
(1′*R*,2*S*)	60.9	125.0	125–25000

directly by the graphic method (log c vs. t). Parameters such as elimination rate constant (β), area under the plasma concentration vs. time curve (AUC^{0-24}), and apparent total clearance (Cl_T/f = dose/AUC^{0-24}) were determined as described previously.[30] Maximum plasma concentration (C_{max}) and the time needed to reach C_{max} (t_{max}) were directly calculated from the plasma isomer concentrations obtained. Renal clearance (Cl_R) was calculated by dividing the amount of each α-hydroxymetoprolol stereoisomer recovered in 24 h urine by its AUC^{0-24}, the apparent eliminated dose fraction (fel/f) was calculated dividing the metoprolol dose corrected by the molecular weight of α-hydroxymetoprolol by the amount of each α-hydroxymetoprolol stereoisomer recovered in 24 h urine and the partial clearance of α-hydroxymetoprolol formation (Cl_M/f) was calculated multiplying the apparent total metoprolol clearance by the apparent eliminated dose fraction of α-hydroxymetoprolol stereoisomers in 24 h.

RESULTS AND DISCUSSION

In the present study we developed methods for the analysis of α-hydroxymetoprolol stereoisomers in human plasma and urine. The analysis was carried out on the chiral column Chiralpak® AD with fluorescence detection.

The analysis of the blank plasma or urine pools collected from different healthy volunteers did not show interference of the endogenous components with the α-hydroxymetoprolol stereoisomers eluted from the chiral stationary phase column (Fig. 2I,II).

The stereochemistry of the new benzylic hydroxylation center was established by Shetty and Nelson,[11] by synthesis and analysis of their CD spectra. A good correlation exists between the sign of the Cotton effect of the ^1L_a band (shorter wavelength transition) and the absolute configuration of phenylcarbinols, which does not vary with the substituent in the aromatic ring. However, the sign of the Cotton effect associated with the ^1L_b and (longer wavelength transition) is highly dependent on the substituent attached to the aromatic ring and on the solvent. The α-hydroxymetoprolol mixture with stereochemistry defined by Shetty and Nelson[11] (1′*S*/1′*R*:70/30) showed in our experiments the same positive CD band at 280 nm observed by the authors. The comparison of the CD spectra of the isolated isomers (Fig. 3) with that of the defined stereochemistry sample, i.e., the (1′*S*)-α-hydroxymetoprolol, allowed to assign the 1′*S* stereoisomers as the less retained on column. Furthermore, taking into account that 2*R*-metoprolol elutes first with respect to the 2*S* one, the following elution order can be proposed: 1′*S*,2*R;* 1′*S*,2*S;* 1′*R*,2*R;* and 1′*R*,2*S* (Fig. 2). Experimental support to this conclusion was obtained by checking the elution order of the α-hydroxymetoprolol stereoisomers on Chiralcel OD. The re-

TABLE 3. Precision and accuracy of the methods for analysis of α-hydroxymetoprolol stereoisomers; within-day precision (n = 10)

Concentration added (ng/ml)	Concentration observed (ng/ml) (mean)	Precision (CV %)	Accuracy (systematic error %)
Plasma 20 ng/mI			
(1′*S*,2*R*)	19.07	1.6	–4.6
(1′*S*,2*S*)	19.04	3.5	–4.8
(1′*R*,2*R*)	19.12	1.1	–4.4
(1′*R*,2*S*)	19.50	3.3	–2.5
Urine 1.0 ng/ml			
(1′*S*,2*R*)	0.95	9.1	–5.0
(1′*S*,2*S*)	0.94	9.9	–6.0
(1′*R*,2*R*)	0.93	9.4	–7.0
(1′*R*,2*S*)	0.95	9.9	–5.0
12.5 ng/ml			
(1′*S*,2*R*)	12.27	9.6	–1.8
(1′*S*,2*S*)	12.89	9.4	3.1
(1′*R*,2*R*)	12.14	10.2	–2.9
(1′*R*,2*S*)	12.29	10.7	–1.7

Systematic error = (concentration observed – concentration added/concentration added) × 100.
CV = coefficient of variation [(SD/mean) × 100].

TABLE 4. Precision and accuracy of the methods for the analysis of α-hydroxymetoprolol stereoisomers; between-day precision (n = 5)

Concentration added (ng/ml)	Concentration observed (ng/ml) (mean)	Precision (CV %)	Accuracy (systematic error %)
Plasma 20 ng/ml			
(1′*S*,2*R*)	19.80	7.9	–1.0
(1′*S*,2*S*)	19.59	9.4	–2.0
(1′*R*,2*R*)	19.74	8.9	–1.3
(1′*R*,2*S*)	19.42	8.3	–2.9
Urine 1.0 ng/ml			
(1′*S*,2*R*)	0.93	5.9	–7.0
(1′*S*,2*S*)	0.93	4.3	–7.0
(1′*R*,2*R*)	0.91	3.9	–9.0
(1′*R*,2*S*)	0.90	5.8	–10.0
12.5 ng/ml			
(1′*S*,2*R*)	12.6	13.5	0.8
(1′*S*,2*S*)	12.8	11.8	2.4
(1′*R*,2*R*)	12.6	14.9	0.8
(1′*R*,2*S*)	12.4	14.2	–0.8

Systematic error = (concentration observed – concentration added/concentration added) × 100.
CV = coefficient of variation [(SD/mean) × 100].

TABLE 5. Study of the selectivity of the methods for the analysis of α-hydroxymetoprolol stereoisomers

Drug	Concentration (μg/ml)	Retention time (min)	
		Plasma	Urine
(1′*S*,2*R*)-α-hydroxy-metoprolol	0.1	15.8	16.5
(1′*S*,2*S*)-α-hydroxy-metoprolol	0.1	16.6	17.9
(1′*R*,2*R*)-α-hydroxy-metoprolol	0.1	20.1	22.2
(1′*R*,2*S*)-α-hydroxy-metoprolol	0.1	24.3	27
(–)-(*S*)-metoprolol	0.05	7.8	7.8
(+)-(*R*)-metoprolol	0.05	6.0	6.3
(±)-N-dealkylmetoprolol	0.1	ND	ND
(±)-O-demethylmetoprolol	0.1	10.4/12.2	10.5/12.5
(±)-Acidic metabolite*	0.1	32	25.3
Benzydamine	0.80	4.2	4.1
Lidocaine	5.00	4.0	3.9
Pindolol	0.20	8.5/10.4	8.5/10.3
Verapamil	0.45	5.8/6.3	5.6/6.0

ND: not detected (0 to 45 min).
*2-hydroxy-3-(4-methoxyethylphenoxy)-propanoic acid.
Drugs not detected (0 to 45 min): acetaminophen, aminopyrine, amiodarone, captopril, carbamazepine, clobazam, clomipramine, clonazepam, chlorpromazine, dapsone, digoxin, disopyramide, etidocaine, metoclopramide, nitrazepam, propafenone, quinidine, theophylline, trimipramine.

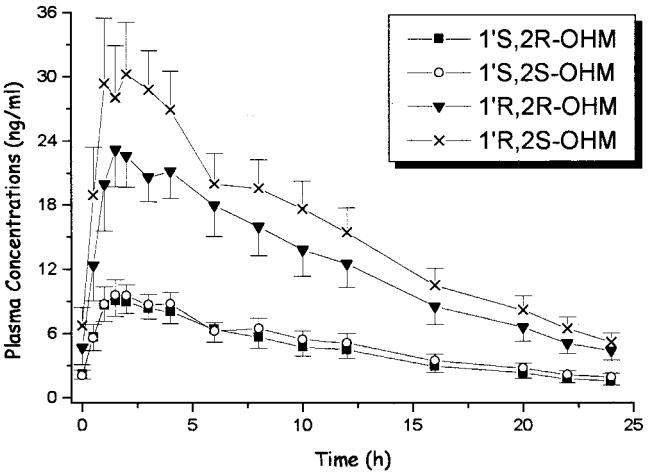

Fig. 4. Plasma concentration vs. time curves for α-hydroxymetoprolol stereoisomers in patients treated with a 200 mg of *rac*-metoprolol tartrate/24 h. Data are reported as mean ± SEM (n = 10).

sults obtained are in agreement with those reported in the literature.[21,26]

Absolute recoveries (Table 2) obtained for α-hydroxymetoprolol stereoisomers by solid-phase extraction of plasma samples were higher than those obtained by liquid–liquid extraction of urine samples (93.7 vs. 63.6%) and independent of the concentrations. Previously reported methods of α-hydroxymetoprolol preferentially employ procedures of liquid–liquid extraction with the use of diethylether,[21] dichloromethane,[5,13,28,31–33] or a mixture of diethyl ether and dichloromethane,[9,17,34,35] with recoveries normally higher than 80%. Balmér et al.[21] achieved a quantitation limit of 14 ng/ml plasma for each isomer. In order to analyze enantiomeric mixture of α-hydroxymetoprolol, solid-phase extraction using C-2 for plasma samples[36] or C-18 columns for urine samples[37] obtained high recovery (more than 80.0%) and low quantitation limit (≤1 ng/ml).

The peak height vs. concentration curves for α-hydroxymetoprolol stereoisomers were constructed in the 5–50 ng/ml plasma or 0.125–25 μg/ml urine range, with correlation coefficients higher than 0.99. The quantitation limits determined by the analysis of 1,000 μl plasma and 100 μl

TABLE 6. Kinetic disposition of α-hydroxymetoprolol stereoisomers after a 200 mg/24 h oral dose of racemic metoprolol tartrate; Mean (CI 95%); n = 10

Parameters	(1′*S*,2*R*)	(1′*S*,2*S*)	(1′*R*,2*R*)	(1′*R*,2*S*)
C_{max} (ng · ml^{-1})	10.15 (7.24–13.05)	10.64 (7.65–13.64)	27.87 (20.35–35.38)	33.68* (22.90–44.45)
t_{max} (h)	2.48 (0.90–4.05)	2.78 (1.17–4.39)	2.68 (1.07–4.28)	2.78 (1.17–4.39)
AUC^{0-24} (ng · h · ml^{-1})	100.97 (66.57–135.38)	113.60 (79.52–147.69)	287.51 (206.17–368.86)	351.91* (243.02–460.79)
$t_{1/2}$ β (h)	7.83 (6.53–9.13)	8.06 (7.08–9.04)	7.70 (6.83–8.57)	7.91 (6.82–9.00)
Fel/f (%)	1.53 (0.88–2.19)	1.84 (0.99–2.70)	4.47 (2.51–6.43)	6.05* (3.06–9.04)
Cl_R (1 · h^{-1} · kg^{-1})	11.99 (8.22–15.75)	11.63 (8.01–15.25)	11.35 (7.94–14.75)	12.08 (8.66–16.94)
Cl_M/f (1 · h^{-1} · kg^{-1})	3.61 (1.15–6.07)	3.98 (1.54–6.42)	7.61 (3.01–12.21)	10.82* (3.57–18.10)
$AUC^{0-24}{}_{2S/2R}$			1.19 (1.06–1.32)	
$AUC^{0-24}{}_{1'R/1'S}$			3.02 (2.86–3.18)	

*$P < 0.05$, Tukey-Kramer multiple comparisons test.

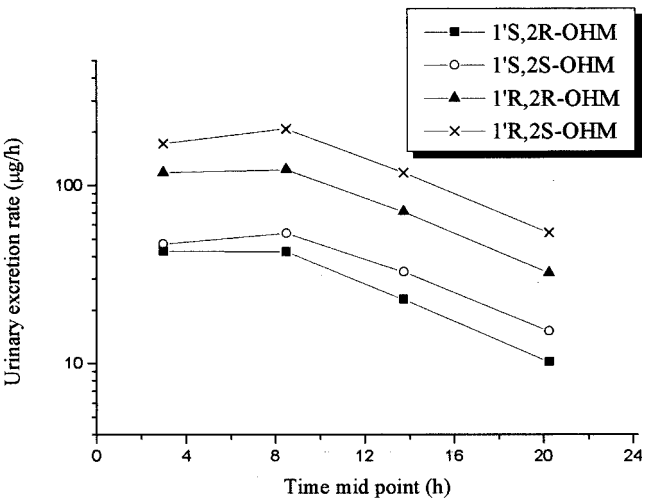

Fig. 5. Urinary elimination (24 h) of α-hydroxymetoprolol stereoisomers in Patient 7 treated with 200 mg of *rac*-metoprolol tartrate/24 h.

urine samples were 2.0 ng/ml and 0.125 µg/ml, respectively, for all α-hydroxymetoprolol stereoisomers analyzed on a Chiralpak® AD column (Table 2).

The coefficients of variation obtained in the study of intra- and interassay precision were less than 15%, assuring the reproducibility and repeatability of the results (Tables 3, 4). The data obtained in the precision study also demonstrated that the internal standard is not required in the analysis.

The analysis of selectivity (Table 5) permitted the application of the direct methods to investigate metoprolol metabolism in clinical studies in combination with other drugs. The metoprolol and the other metabolites also did not interfere with the analysis.

The methods developed and validated were employed in the investigation of stereoselectivity in the metabolism of metoprolol administered p.o. in the racemic form in a multiple dose regimen (200 mg dose of the tartrate salt/24 h) to patients phenotyped as extensive metabolizers of debrisoquine.[29]

The plasma concentration vs. time curves for the four α-hydroxymetoprolol stereoisomers are presented in Figure 4 as means ± SEM (n = 10). The plasma concentrations for the 1′*R* stereoisomers were significantly higher ($p < 0.05$) than those of 1′*S* stereoisomers, resulting in a mean ratio $AUC^{0-24}{}_{1'R}/AUC^{0-24}{}_{1'S}$ of 3.02, with the 2*S* stereoisomers being the metabolites found in slightly greater amount than the 2*R* (Table 6). There are no literature data about the α-hydroxymetoprolol stereoisomers in plasma. Murthy et al.[9] reported mean urinary excretion ratio $Ae^{0-24}{}_{1'R}/Ae^{\circ 0-24}{}_{1'S}$ of 3.12 in studies of a single oral dose of racemic metoprolol in volunteers. No significant differences were observed between the stereoisomers in the terminal half-life ($t_{1/2}\beta$) and renal clearance (Cl_R) (Table 6; Fig. 5). The absence of stereoselectivity in renal excretion indicates that the higher plasma concentrations of the 1′*R* stereoisomers result from the preferential formation of this new chiral center. Considering the slightly greater amount of (2*S*)-α-hydroxymetoprolol stereoisomers, we can conclude that this pathway is not responsible for the plasma accumulation of (–)-(*S*)-metoprolol in humans.[8]

ACKNOWLEDGMENTS

The authors are grateful to FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) and to MIUR (Roma, Italy) for financial support. The authors are also grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for granting research fellowships, and the CNR/CNPq bilateral agreement for supporting the exchange of researchers.

LITERATURE CITED

1. Wahlund G, Nerme V, Abrahamsson T, Sjoquist P-O. The β_1- and β_2-adrenoceptor affinity and β_1- blocking potency of S- and R-metoprolol. Br J Pharmacol 1990;99:592–596.
2. Regardh C-G, Johnsson G. Clinical pharmacokinetics of metoprolol. Clin Pharmacok 1980;5:557–569.
3. Johnson JA, Burlew BS. Metoprolol metabolism via cytochrome P4502D6 in ethnic populations. Drug Metab Dispos 1996;24:350–355.
4. Jonkers RE, Koopmans RP, Portier EJG, Boxtel CJ. Debrisoquine phenotype and the pharmacokinetics and *beta*-2 receptor pharmacodynamics of metoprolol and its enantiomers. J Pharmacol Exp Ther 1991;256: 959–966.
5. Lennard MS, Tucker GT, Silas JH, Freestone S, Ramsay LE, Woods HF. Differential stereoselective metabolism of metoprolol in extensive and poor debrisoquine metabolizers. Clin Pharmacol Ther 1983;34: 732–737.
6. Andersson T, Lundborg P, Regardh CG. Lack of effect of omeprazole treatment on steady-state plasma levels of metoprolol. J Clin Pharmacol 1991;40:61–65.
7. Laurent-Kenesi M-A, Funck-Brentano C, Poirier J-M, Decolin D, Jaillon P. Influence of CYP2D6-dependent metabolism on the steady-state pharmacokinetics and pharmacodynamics of metoprolol and nicardipine, alone and in combination. Br J Clin Pharmacol 1993;36:531–538.
8. Cerqueira PM, Cesarino EJ, Mateus FE, Mere Y Jr, Santos SRCJ, Lanchote VL. Enantioselectivity in the steady-state pharmacokinetics of metoprolol in hypertensive patients. Chirality 1999;11:591–597.
9. Murthy SS, Shetty HU, Nelson WL, Jackson PR, Lennard MS. Enantioselective and diastereoselective aspects of the oxidative metabolism of metoprolol. Biochem Pharmacol 1990;40:1637–1644.
10. Genko FM, Ulatowski JA, McHugh WB. Metoprolol and alpha-hydroxymetoprolol concentrations and reduction in essential tremor. Clin Pharmacol Ther 1984;36:320–325.
11. Shetty HU, Nelson WL. Chemical aspects of metoprolol metabolism. Asymmetric synthesis and absolute configuration of the 3-[4-(1-hydroxy-2-methoxyethyl)phenoxy]-1-(isopropylamino)-2-propanols, the diastereomeric benzylic hydroxylation metabolites. J Med Chem 1998; 31:55–59.
12. Otton SV, Crewe HK, Lennard MS, Tucker GT, Woods HF. Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P450 in metoprolol oxidation by human liver microsomes. J Pharmacol Exp Ther 1988;247:242–247.
13. Lennard MS. Quantitative analysis of metoprolol and three of its metabolites in urine and liver microsomes by high-performance liquid chromatography. J Chromatogr 1985;342:199–205.
14. Walhagen A, Edholm L-E. Coupled-column chromatography on immobilized protein phases for direct separation and determination of drug enantiomers in plasma. J Chromatogr 1989;473:371–379.
15. Balmér K, Persson B-A, Schill G. Optimization of detection sensitivity for enantiomers of metoprolol on silica-bonded α_1-acid glycoprotein. J Chromatogr 1989;477:107–119.
16. Enquist M, Hermansson J. Separation of the enantiomers of β-blocking agents and other cationic drugs using a CHIRAL-AGP® column. Bind-

ing properties and characterization of immobilized α_1-acid glycoprotein. J Chromatogr 1990;519:285–298.

17. Persson B-A, Balmér K, Lagerstrom P-O, Schill G. Enantioselective determination of metoprolol in plasma by liquid chromatography on a silica-bonded α_1-acid glycoprotein column. J Chromatogr 1990;500: 629–636.
18. Ching MS, Lennard MS, Gregory A, Tucker GT. Measurement of underivatised enantiomers in human plasma by high-performance liquid chromatography with a chiral stationary phase. J Chromatogr 1989; 497:313–318.
19. Rutledge DR, Garrick C. Rapid high-performance liquid chromatographic method for the measurement of the enantiomers of metoprolol in serum using chiral stationary phase. J Chromatogr 1989;497:181–190.
20. Straka RJ, Johnson KA, Marshall PS, Remmel RP. Analysis of metoprolol enantiomers in human serum by liquid chromatography on a cellulose-based chiral stationary phase. J Chromatogr 1990;530:83–93.
21. Balmér K, Persson A, Lagerstrom P-O, Persson B-A, Schill G. Liquid chromatographic separation of the enantiomers of metoprolol and its α-hydroxy metabolite on Chiralcel OD for determination in plasma and urine. J Chromatogr 1991;553:391–397.
22. Herring VL, Bastian TL, Lalonde RL. Solid-phase extraction and direct high-performance liquid chromatographic determination of metoprolol enantiomers in plasma. J Chromatogr 1991;567:221–227.
23. Leloux MS. Rapid chiral separation of metoprolol in plasma-application to the pharmacokinetics/pharmacodynamics of metoprolol enantiomers in the conscious goat. Biomed Chromatogr 1992;6:99–105.
24. Lanchote VL, Bonato PS, Cerqueira PM, Pereira VA, Cesarino EJ. Enantioselective analysis of metoprolol in plasma using high-performance liquid chromatographic direct and indirect separations: applications in pharmacokinetics. J Chromatogr B 2000;738:27–37.
25. Kim KH, Kim HJ, Kang JS, Mar W. Determination of metoprolol enantiomers in human urine by coupled achiral-chiral chromatography. J Phar Biomed Anal 2000;22:377–384.
26. Kim KH, Shin SD, Lee JH, Lee SC, Kang JS, Mar W, Hong SP, Kim HJ. Chiral separation of the enantiomers of metoprolol and its metabolites by high performance liquid chromatography. Arch Pharm Res 2000; 23:230–236.
27. Mistry B, Leslie JL, Eddington ND. Enantiomeric separation of metoprolol and α-hydroxymetoprolol by liquid chromatography and fluorescence detection using chiral stationary phase. J Chromatogr B Biomed Sci Appl 2001;758:153–161.
28. Schuster D, Modi MW, Lalka D, Gengo FM. Reversed-phase high-performance liquid chromatographic assay to quantitate diastereomeric derivatives of metoprolol enantiomers in plasma. J Chromatogr 1988;433:318–325.
29. Cerqueira PM, Mateus FH, Cesarino EJ, Bonato BS, Lanchote VL. Enantioselectivity of debrisoquine 4-hydroxylation in Brazilian Caucasian hypertensive patients phenotyped as extensive metabolizers. J Chromatogr B 2000;749:153–161.
30. Shargel L, Yu ABC. Applied biopharmaceutics and pharmacokinetics. London: Prentice Hall International; 1993.
31. Pautler DB, Jusko WJ. Determination of metoprolol and α-hydroxymetoprolol in plasma by high-performance liquid chromatography. J Chromatogr 1982;228:215–222.
32. Gengo FM, Ziemniak MA, Kinkel WR, McHugh WB. High-performance liquid chromatographic determination of metoprolol and α-hydroxymetoprolol concentrations in human serum, urine and cerebrospinal fluid. J Pharm Sci 1984;73:961–963.
33. Horai Y, Ishizaki T, Kusaka M, Tsujimoto G, Hashimoto K. Simultaneous determination of metoprolol and α-hydroxymetoprolol in human plasma and urine by liquid chromatography with a preliminary observation on metoprolol oxidation in Japanese subjects. Ther Drug Monit 1988;10:428–433.
34. Lecaillon J-B, Godbillon J, Abadie F, Gosset G. Determination of metoprolol and its α-hydroxylated metabolite in human plasma by high-performance liquid chromatography. J Chromatogr 1984;305:411–417.
35. Ahnoff M, Chen S, Green A, Grundevik I. Chiral chloroformates as transparent reagents for the resolution of metoprolol enantiomers by reversed-phase liquid chromatography. J Chromatogr 1990;506:593–599.
36. Mistry B, Leslie J, Eddington NE. A sensitive assay of metoprolol and its major metabolites α-hydroxy metoprolol in human plasma and determination of dextromethorphan and its metabolite dextrorphan in urine with high performance liquid chromatography and fluorometric detection. J Pharm Biomed Anal 1998;16:1041–1049.
37. Chiu FCK, Damani LA, Li RC, Tomlinson B. Efficient high-performance liquid chromatographic assay for the simultaneous determination of metoprolol and two main metabolites in human urine by solid-phase extraction and fluorescence detection. J Chromatogr B 1997;696:69–74.