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Stereoselective analysis of metoprolol and its metabolites in rat plasma with application to oxidative metabolism

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Abstract

We investigated the stereoselective kinetic disposition and metabolism of metoprolol (MET) in rats. The racemic MET (15 mg/kg) was given by oral gavage and blood samples were collected from 0 to 10 h ($n = 6$ at each time point). The enantiomeric concentrations of MET and its metabolites α-hydroxymetoprolol (α-OHM) and *O*-demethylmetoprolol (ODM) were determined by HPLC using a Chiralpak® AD chiral column and fluorescence detection. The pharmacokinetic parameters of unchanged MET and the formation of ODM did not show to be stereoselective. In contrast, the AUC (ng h/mL) of α-hydroxymetoprolol isomers were higher to I′*R* [638.2(525.2–706.2) for 1′R2R and 659.6(580.4–698.1) for 1′*R*,2S, mean, (95%CI)] than to I′*S* products [58.3(47.4–66.1) for 1′*S*,2R and 57.1(44.7–67.9) for 1′*S*,2*S*, mean, (95%CI)]. We conclude that the kinetic disposition of unchanged MET and the formation of ODM are not enantioselective in rats but the metabolism of α-OHM yields predominantly the 1′*R*-product.

Keywords: Metoprolol; Enantiomers; Metabolism; Rats; Pharmacokinetics; CYP2D6

1. Introduction

Metoprolol (MET) is a selective β_1-adrenoceptor antagonist used as a racemic mixture for the treatment of hypertension and ischemic heart disease [1–3]. The (*S*)-(−)-MET enantiomer has significantly greater β_1-adrenergic receptor affinity by 25-fold [4–6].

There are three main oxidative metabolic pathways involved in 85% of the MET metabolism in man: *O*-demethylation with subsequent fast oxidation, aliphatic hydroxylation, and oxidative deamination [6], which form the correspondent metabolites *O*-demethylmetoprolol (ODM), metoprolol acidic metabolite (AODM), *N*-dealkylmetoprolol (*N*-DAM), and α-hydroxymetoprolol (α-OHM) (Fig. 1).

MET is metabolized in the rat by same pathways observed in man. However, the *N*-DAM metabolite is found in considerably lower relative amounts in the rat urine than in man urine [7]. In rat liver microsomal preparation the rate of α-hydroxylation exceeded *O*-demethylation by about 30%. However, in the human liver microsomal preparations, *O*-demethylation exceeded α-hydroxylation by about 7- to 12-fold [6].

A moderate enantioselectivity of MET pharmacokinetics has been reported in humans [8,9]. Mostafavi and Foster [10] observed that the pharmacokinetics of metoprolol is stereoselective in rats after oral administration of racemate. Slightly but significantly greater amounts of the *R*-(+)-MET are observed in rat plasma after a *p.o.* dose. Consequently, oral clearance values are higher for the *S*-(−) enantiomer (2.26 L/min/kg) than for the *R*-(+) enantiomer (1.99 L/min/kg). The elimination half-life ($t_{1/2}$) of MET is similar (35 min) for both the *R*-(+) and *S*-

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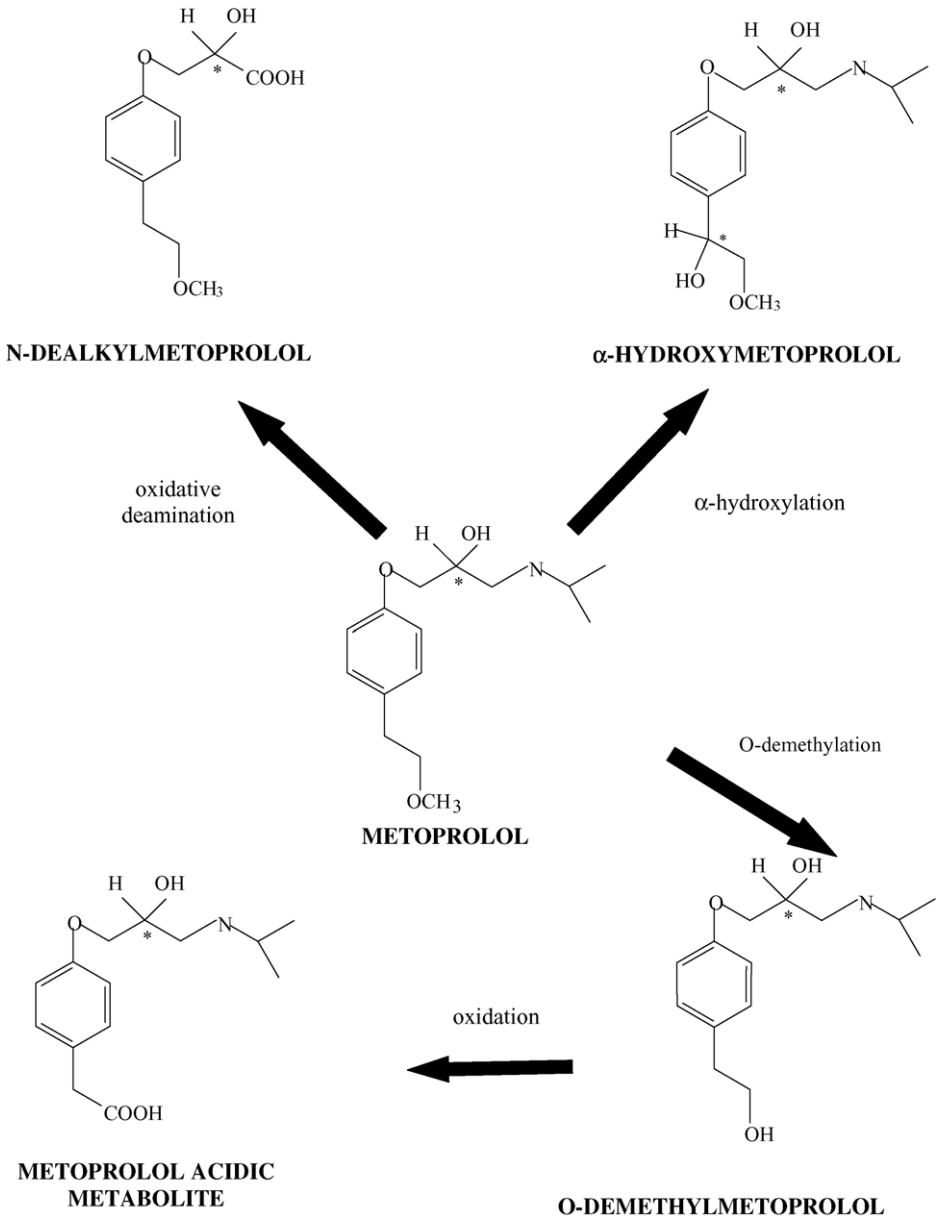

Fig. 1. Oxidative metabolism of metoprolol.

(−) enantiomers, as observed in human extensive MET metabolizers.

The α-hydroxylation pathway shows a high degree of product stereoselectivity in rat liver microsomes, favoring formation of a new 1′*R* chiral center from both enantiomers of metoprolol [6,11]. However, the complete in vivo study of stereoselective MET metabolism in both rat and human has not been done.

Most chromatographic separations of enantiomers are classified into two general categories, i.e., indirect and direct methods of separation. Both methods have been applied to the separation of metoprolol isomers [1,12–14]. Recently, chiral chromatography methods have been developed for metoprolol enantiomers in plasma and urine [15,16]. Mistry et al. [17] reported the determination of metoprolol enantiomers and the two pairs of α-hydroxymetoprolol diastereoisomers in plasma by liquid chromatography and fluorescence detection using a Chirobiotic T bonded phase column. Regarding the separation of enantiomers MET metabolites, Cerqueira et al. [3] developed a method that quantifies metoprolol enantiomers and the four stereoisomers of α-hydroxymetoprolol isomers simultaneously in plasma and urine. However, only Murthy et al. [6] developed an enantioselective method for analysis of ODM. The metabolite was derivatized with phosgene and bistrimethylsilyltrifluoroacetamide and analyzed by GC–MS. No method is available for direct enantioselective analysis of ODM by HPLC.

In view of the absence of in vivo studies on stereoselective metabolism of MET, we report the development, validation and application of the HPLC method of sequential analysis of the MET, ODM and α-OHM isomers in rat plasma.

2. Materials and methods

2.1. Chemicals

(±)-MET tartrate was obtained from Sigma (St. Louis, MO, USA). The MET metabolites H119/66 as *p*-OH benzoate (α-OHM) and H105/22 as *p*-OH benzoate (ODM) were kindly donated by Astra Hässle AB (Mölndal, Sweden). Solvents used to prepare the samples and mobile phases were HPLC grade from Merck (Darmstadt, Germany). Other reagents were analytical grade. Water was purified with a Milli-Q Plus ultra-pure water system (Millipore, Bedford, USA).

Sodium chloride from Merck and sodium hydroxide solutions were washed twice with diisopropyl ether–dichloromethane 1:1 (v/v).

2.2. Apparatus

The HPLC system consisted of a model SIL-10ADVP auto-injector with temperature set as 5 °C, a SIL-10ADVP pump for isocratic elution, a RF551 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 274 nm was used to determine the optical rotation of *O*-demethylmetoprolol enantiomers and the UV and CD chromatograms were obtained simultaneously.

The chiral column used was an amylose Tris (3,5-dimethylphenyl carbamate) coated on a 10 μm silica gel substrate (Chiralpak AD, 250 mm × 4.6 mm), from Daicel Chemical Industries (New York, EUA) with a 4 mm × 4 mm Lichrospher 100 CN precolumn, 10 μm particle size (Merck).

2.3. Standard solutions

Standard solutions of (±)-MET tartrate as well as MET metabolites were prepared in methanol at 0.2 mg free base/mL. The diluted solutions were prepared at concentrations of 0.8, 1.0, 1.6, 2.0, 4.0, 8.0, 10, 20, 40, 80 and 100 μg/mL methanol and stored at −20 °C in the dark.

2.4. Sample preparation

Plasma samples (1.0 mL) were alkalinized with 250 μL of a 1M sodium hydroxide aqueous solution. Sodium chloride (100 mg) and 4.0 mL diisopropyl ether–dichloromethane (1:1, v/v) were added. Samples were shaken for 30 min in a horizontal shaker (220 ± 10 cycles/min) and centrifuged at 1800 × *g* for 5 min and the organic phases (3.5 mL) were transferred to conical tubes and evaporated to dryness in an evaporator system consisting of a Centrifugal Evaporator RC 10.22, Jouan (Saint Herblain, France) and a Refrigerated Trap RCT.90, Jouan (Saint Herblain, France). The residues obtained were dissolved in 150 μL of the mobile phase and 100 μL injected into the chromatographic system.

2.5. Chromatography

Separation of the isomers was performed on a Chiralpak® AD column with the mobile phase consisting of a mixture of hexane:ethanol:isopropanol:diethylamine (88:10.2:1.8:0.2, v/v/v/v). The flow rate was 1.2 mL/min.

2.6. Identification of O-ODM enantiomers

To determine the optical rotation of each ODM enantiomer, 400 μL of a 5 μg/mL standard solution were evaporated to dryness, the residue was dissolved in 25 μL mobile phase and 20 μL injected into the chromatographic system described in Section 2.2, using the circular dichroism detector. The results were compared with the CD spectra of pure MET enantiomers. The elution order of MET, α-OHM and the acidic metabolite was determined previously [3,18].

2.7. Calibration curves and validation of the methods

The calibration curves were constructed from 1.0 mL samples of rat blank plasma spiked with 25 μL of each diluted standard solution of MET and metabolites. The linear regression equations and the correlation coefficients were obtained from the heights of the peaks plotted against their respective plasma concentration (10–250 ng/mL for each MET and ODM enantiomer and 5–125 ng/mL of α-OHM isomer).

The recovery of MET and its metabolites was evaluated by comparing the heights of the peaks obtained after plasma extraction with the heights of the peaks obtained after injection of the standard solutions. Standard solutions (25 μL) were evaporated to dryness, the residues obtained were dissolved in the mobile phase (100 μL) and injected (100 μL) into the chromatographic system.

The interference of the other MET metabolites was evaluated by the analysis of rat blank plasmas spiked with their standard solutions.

The quantitation limit (LOQ) was obtained by the analysis in quintuplicate of plasma samples spiked with MET, ODM and α-OHM at concentrations as low as 1 ng/mL for MET and ODM and 5 ng/mL for α-OHM. The LOQ was defined as the lowest plasma concentration of each analyte analysed with an error of 20% or lower.

The linearity was studied by the analysis of plasma samples spiked with the increasing analyte 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 and accuracy of the method were evaluated by analyzing MET, ODM and α-OHM in plasma samples spiked with three concentrations, 10, 50 and 100 ng/mL, for the MET and ODM enantiomers and 12.5, 50 and 100 ng/mL of each isomer of α-OHM. Aliquots of spiked plasma samples were stored at −20 °C and analyzed in replicate experiments ($n = 10$) using a single calibration curve for intra-assay evaluation, and in duplicate on five consecutive days for inter-assay evaluation.

The stability was assured by three freeze (−70 °C) and thaw (24 °C) cycles and long- term stability. Spiked plasma samples were prepared and an aliquot was assayed on the same day. Another aliquot was kept at −70 °C for 30 days and then assayed. This period corresponds to the maximum time the samples were kept frozen.

The risk of MET, ODM and α-OHM racemization was analysed by spiking rat blank plasma with isolated isomers and the isomeric purity was evaluated after extraction.

2.8. Metoprolol acidic metabolite enantiomers in plasma

Metoprolol acidic metabolite enantiomers in plasma were analysed as previously described by our group [18].

2.9. Experimental sampling

2.9.1. Animal maintenance

The study was conducted on male Wistar rats (220–250 g) provided by the University of São Paulo. Three days before the experiments the animals were brought to the local animal room, where temperature and humidity were controlled and the dark–light cycle were set at 12 h. Water and food were offered ad libitum until 12 h before the animals received the drug.

The experiments were carried out in accordance with the recommendations of the Ethics Committee on Animal Research of the Federation of Brazilian Societies of Experimental Biology.

2.9.2. Dosing and sample collection

The method was applied to the investigation of stereoselectivity in the kinetic disposition and metabolism of MET administered in the racemic form in a single dose to rats. After a 12 h fast, the animals received an aqueous solution of rac-MET tartrate (15 mg/kg) by gavage [19]. Heparinized blood samples were collected by decapitation at times 0, 2, 4, 6, 8, 10, 20, 30, 40 and 50 min and 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 and 10 h after drug administration. Six rats were employed for each time. Plasma samples were kept at −70 °C until analyses.

2.10. Pharmacokinetics

The pharmacokinetic parameters were obtained by fitting the data to a bicompartmental open model for MET and a monocompartmental model for all three metabolites. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from the raw data. Terminal half-lives ($t½\beta$)) were determined by linear regression of the later part of the plasma concentration versus time curve of individual enantiomers. The rate constants (Kel or β) were calculated using the $0.693/t^1/_2$ equation. The areas under the plasma concentration *versus* time curves ($AUC^{0-\infty}$) were calculated by the trapezoid method with infinite extrapolation. This parameter was used for the calculation of apparent total clearance ($Cl_T/f = dose/AUC^{0-\infty}$) and apparent volume of distribution ($Vd/f = Cl/f/\beta$) where β is the elimination rate constant [20].

2.11. Statistical analysis

The statistical tests for the calculation of the median, mean, SEM and 95% confidence interval (95% CI) were performed using the GraphPad Instat GraphPad Software. The Wilcoxon test was used for the determination of enantiomer ratios different from one for MET and ODM, and the Kruskal–Wallis test was used for α-OHM. Statistical significance was set at $p < 0.05$.

3. Results and discussion

The present study describes for the first time the complete stereoselective metabolism of MET in rats. For this, we developed and validated a fast, sensitive and simple HPLC method for the stereoselective analysis of MET, α-OHM and ODM in rat plasma. To the best of our knowledge, this is the only HPLC stereoselective assay that can separate MET, ODM and α-OHM (Fig. 2). The ODM metabolite is not detected in human plasma but is detected in the plasma of rats treated with racemic MET at the dose of 15 mg/kg weight. As a complementary result, in addition to the data for the α-OHM and ODM metabolites, we also present the data regarding the isomers of AODM in rat plasma.

The order of elution of the ODM enantiomers was determined with a circular dichroism detector. Fig. 3 shows that the first enantiomer eluted from the Chiralpak® AD column presented a positive sign and the second enantiomer presented a negative sign in the chromatogram of circular dichroism at 274 nm. The spectra of the pure MET enantiomers presented a positive sign at 274 nm for (*R*)-(+)-MET and a negative sign at the same wavelength for (*S*)-(−)-MET [18]. Because of the small difference in chemical structure between ODM and MET, with no change in the chiral center, we may assume that the ODM enantiomers have same absolute configuration, determining the order of elution as (*R*)-(+)-ODM and (*S*)-(−)-ODM. The order of elution of the MET enantiomers and the α-OHM isomers was established as previously reported by Cerqueira et al. [18].

The analysis of blank plasma pools collected from different rats did not show interference of the endogenous

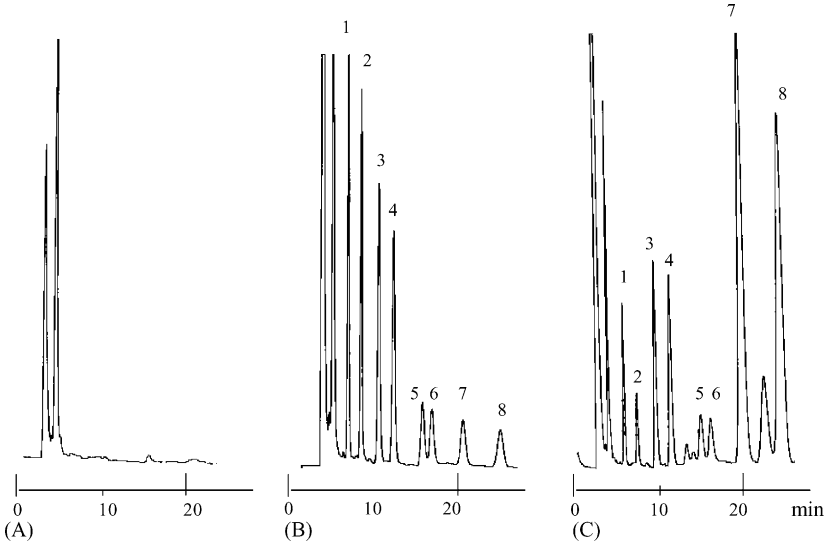

Fig. 2. Chromatograms referring to the analysis of the metoprolol and *O*-demethylmetoprolol enantiomers and of the α-hydroxymetoprolol isomers in plasma: (A) blank; (B) plasma enriched with racemic metoprolol and *O*-demethylmetoprolol (25 ng of each enantiomer/mL plasma) and α-hydroxymetoprolol (12.5 ng of each isomer/mL plasma); (C) rat plasma sample obtained 2 h after metoprolol administration. (1) *R*-(+)-metoprolol; (2) *S*-(−)-metoprolol; (3) *R*-(+)-*O*-demethylmetoprolol; (4) *S*-(−)-*O*-demethylmetoprolol; (5) 1′*S*,2*R*-α-hydroxymetoprolol; (6) 1′*S*,2*S*-α-hydroxymetoprolol; (7) 1′*R*,2*R*-α-hydroxymetoprolol; and (8) 1′*R*,2*S*-α-hydroxymetoprolol.

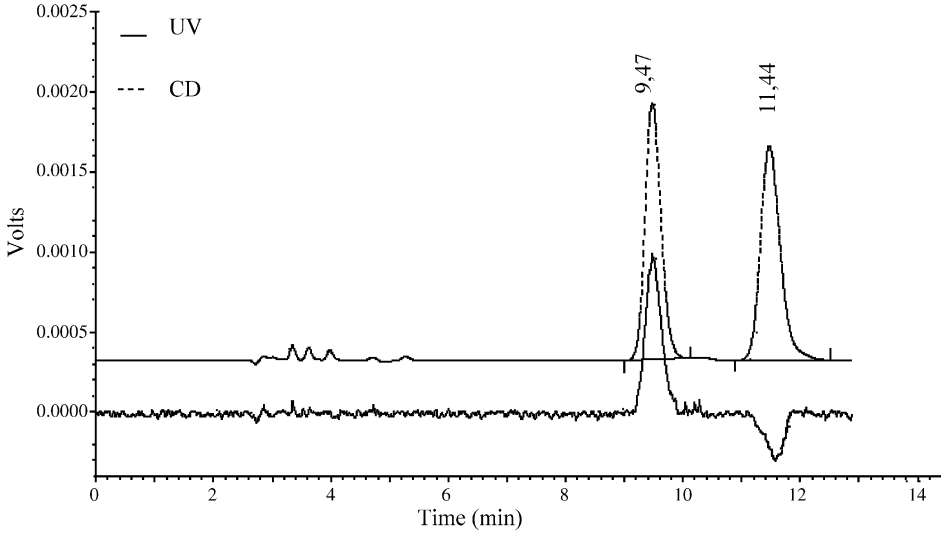

Fig. 3. Chromatograms referring to the analysis of circular dichroism (CD) of the *O*-demethylmetoprolol enantiomers. (*R*)-(+)-(9.47 min; positive peak in CD) and (*S*)-(−)-*O*-demethylmetoprolol (11.44 min; negative peak in CD).

components with the MET and its metabolites eluted from the chiral stationary phase column.

The peak height versus concentration curves for MET and its metabolites were constructed in the 1–2500 ng/mL plasma range for MET and ODM and in the 5–1250 ng/mL plasma range for α-OHM, with correlation coefficients higher than 0.99. The quantitation limits determined by the analysis of 1 mL plasma were 1 ng/mL for MET and O-ODM and 5 ng/mL for α-OHM (Table 1).

The coefficients of variation obtained in the study of intra- and interassay precision and accuracy were less than 10% (10–100 ng of each enantiomer/mL for MET and ODM and 12.5–100 ng of each isomer/mL for α-OHM), assuring the

Table 1
Confidence limits of the method for the analysis of MET, ODM and α-OHM in plasma

	Recovery (%)	Quantitation limit (ng/mL) (CV, %)	Linearity (ng/mL)
R-(+)-MET	90.7	1.0 (9.0)	1–2500
S-(−)-MET	88.4	1.0 (11.7)	1–2500
R-(+)-ODM	84.3	1.0 (9.0)	1–2500
S-(−)-ODM	82.7	1.0 (11.7)	1–2500
1′*S*,2*R*-OHM	75.3	5.0 (10.1)	5–1250
1′*S*,2*S*-OHM	75.9	5.0 (12.5)	5–1250
1′*R*,2*R*-OHM	73.3	5.0 (9.7)	5–1250
1′*R*,2*S*-OHM	74.7	5.0 (8.9)	5–1250

CV: coefficient of variation [(S.D./mean) × 100].

Table 2
Study of the stability of the method for analysis of the MET and ODM enantiomers and of the α-OHM isomers in plasma

Concentration	Short-term (3 cycles)		Long-term (30 days)	
	Precision (CV %)	Accuracy (% inaccuracy)	Precision (CV %)	Accuracy (% inaccuracy)
MET (100 ng/mL)				
R-(+)-MET	3.7	+4.5	7.6	−5.6
S-(−)-MET	4.8	+3.9	6.4	−6.8
ODM (100 ng/mL)				
R-(+)-ODM	3.1	−2.3	3.1	−5.1
S-(−)-ODM	2.5	−3.1	4.5	−7.8
α-OHM (50n g/ML)				
1′*S*,2*R*	4.5	+4.7	5.8	+4.9
1′*S*,2*S*	4.9	+5.1	7.5	−5.3
1′*R*,2*R*	5.1	−7.2	6.8	+7.8
1′*R*,2*S*	3.1	−8.9	8.3	+6.7

CV: coefficient of variation [(S.D./mean) × 100]; %Systematic error = [(concentration observed-concentration added)/concentration added] × 100.

Table 3
Enantioselective kinetic disposition of MET in rat plasma

	R-(+)-MET	*S*-(−)-MET
C_{max} (ng/mL)	450.0 451.0 (418.4–483.6)	513.4* 512.2 (476.2–548.2)
t_{max} (h)	0.06 0.07 (0.02–0.12)	0.06 0.07 (0.02–0.12)
$AUC^{0-\infty}$ (ng h/mL)	101.0 98.0 (72.2–123.8)	83.4 78.2 (57.1–99.3)
Vd/f (L/kg)	53.8 67.7 (36.1–99.3)	66.5 70.9 (37.0–104.8)
Cl_T/f (L/h/kg)	76.0 76.0 (58.6–103.3)	90.2 102.0 (71.6–132.4)
$t_{\frac{1}{2}}$ β (h)	0.57 0.57 (0.43–0.71)	0.49 0.48 (0.34–0.62)
β (h^{-1})	1.23 1.29 (0.93–1.65)	1.41 1.57 (1.03–2.12)
$AUC^{0-\infty}_{(S)-(-)/(R)-(+)}$	0.76 0.80 (0.67–0.94)	

Median, mean and (CI 95%), $n = 6$ for each time.
* $p < 0.05$, Wilcoxon test.

reproducibility and the repeatability of the results. The data obtained in the precision and accuracy studies also demonstrated that the internal standard is not required in the analysis.

The tests of short- and long-term stability insured the good condition of the samples during freezing, from collection to analysis, with no variation of more than 10% in either short- or long-term stability at any of the concentrations tested (Table 2).

The developed and validated method was used in the study of enantioselectivity in the kinetic disposition of MET administered by gavage in the racemic form in a single 15 mg/kg dose of the tartrate salt to rats. For the complete investigation of MET metabolism, we analysed the metoprolol acidic metabolite as previously described by Cerqueira et al. [18]. Thus, in this present work, we extend the previous in vitro data reported by Murthy et al. [6] showing the stereoselective aspects of the oxidative metabolism of MET in a rat model.

The pharmacokinetics of MET is not enantioselective ($p > 0.05$) for the parameters $AUC^{0-\infty}$, t_{max}, Vd, Cl and $t_{1/2}$

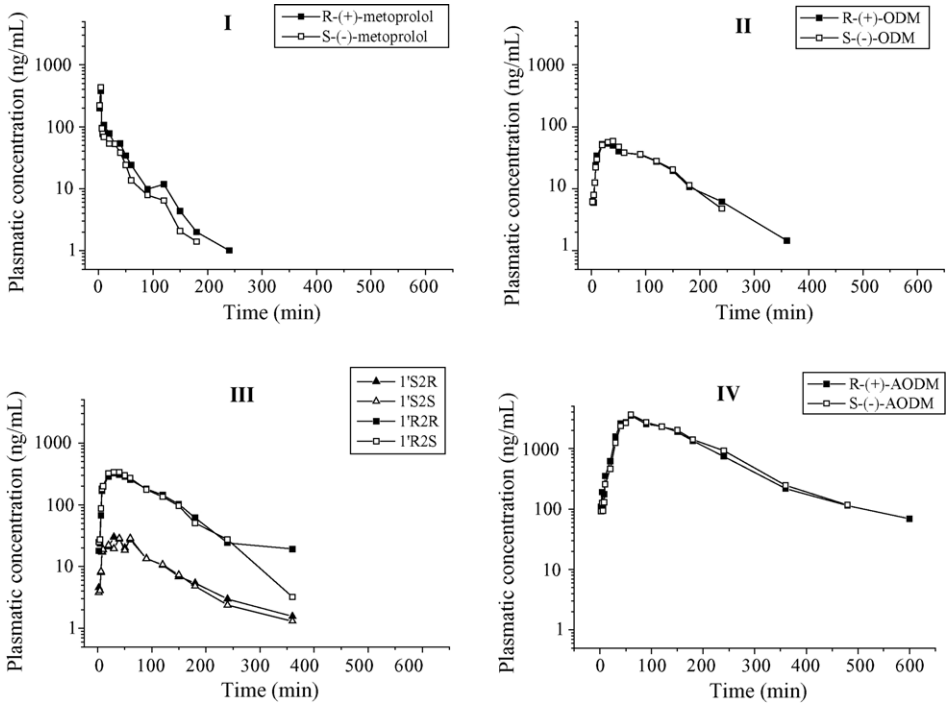

Fig. 4. Plasma concentration vs. time curves for metoprolol and its metabolites in rat plasma: (I) MET enantiomers; (II) ODM enantiomers; (III) α-OHM isomers; and (IV) AODM enantiomers.

Table 4
Enantioselective kinetic disposition of ODM and AODM in rat plasma

	(*R*)-(+)-ODM	(*S*)-(−)-ODM	(*R*)-(+)-AODM	(*S*)-(−)-AODM
C_{max} (ng/mL)	55.6 56.0 (46.5–65.6)	62.0 62.2 (50.3–74.2)	3253.8 3144.9 (2140.7–4149.1)	3282.2 3216.6 (2298.9–4134.1)
t_{max} (h)	0.66 0.60 (0.52–0.69)	0.66 0.60 (0.52–0.69)	1.00 0.97 (0.89–1.05)	1.00 0.97 (0.89–1.05)
$AUC^{0-\infty}$ (ng h/mL)	121.2 118.8 (98.5–139.1)	126.4 131.1 (103.8–158.4)	8319.0 8579.1 (7363.2–9795.0)	9596.2 9814.3 (8388.9–11240.0)
$t\frac{1}{2}$ (h)	1.46 1.34 (1.09–1.59)	1.28 1.26 (0.97–1.55)	1.53 1.61 (1.24–1.98)	1.43 1.49 (1.11–1.87)
Kel (h^{-1})	0.47 0.53 (0.41–0.65)	0.56 0.57 (0.44–0.70)	0.45 0.45 (0.35–0.54)	0.52 0.51 (0.42–0.61)
$AUC^{0-\infty}_{(S)-(-)/(R)-(+)/}$	1.11 1.11 (0.93–1.28)		1.11 1.16 (0.99–1.34)	

Median, mean and (CI 95%), $n = 6$ for each time; $^{*}p < 0.05$, Wilcoxon test.

(Fig. 4I, Table 3). However, C_{max} was higher for *S*-(−)-MET (513.45 ng/mL) than *R*-(+)-MET (450.00 ng/mL) (Table 3). These data agree with those reported by Mostafavi et al. [10] in an investigation of enantioselectivity in the pharmacokinetics of unchanged MET orally administered to rats in a single or multiple dose. Area under the curve (AUC for plasma concentration versus time) values between *R*- and *S*-MET were not significantly different ($p > 0.05$) suggesting that there is no difference in enantiomers bioavailability. The bioavailability of MET in the racemic form is 4% in rats [7], however the bioavailability of the individual MET enantiomers was not previously reported.

The formation of the ODM metabolite and the subsequent formation of AODM did not show enantioselectivity since AUC *S*/*R* ratios close to 1 were obtained for both metabolites; (Table 4).

From a quantitative point of view, the main metabolite present in rat plasma was AODM since the $AUC^{0-\infty}$ AODM/MET ratio was approximately 97. *O*-Demethylation was less important quantitatively, and the $AUC^{0-\infty}$ for the ODM/MET ratio was 1.4 (Tables 3 and 4).

For α-OHM there was a statistically significant difference ($p < 0.05$), with a predominance of the formation of hydroxylated 1′*R* compounds over 1′*S* compounds ($AUC^{0-\infty}$

Table 5
Enantioselective kinetic disposition of α-OHM in rat plasma

	1′*S*,2*R*-OHM	1′*S*,2*S*-OHM	1′*R*,2*R*-OHM	1′*R*,2*S*-OHM
C_{max} (ng/mL)	36.3[a,b] 38.8 (26.6–50.9)	32.1[c,d] 32.0 (24.6–39.7)	336.2 342.9 (304.7–381.1)	360.6 364.4 (304.6–424.2)
t_{max} (h)	0.53 0.61 (0.39–0.82)	0.58 0.58 (0.49–0.67)	0.63 0.64 (0.43–0.84)	0.58 0.58 (0.34–0.82)
$AUC^{0-\infty}$ (ng h/mL)	58.3[a,b] 56.7 (47.4–66.1)	57.1[c,d] 56.3 (44.7–67.9)	638.2 615.7 (525.2–706.2)	659.6 639.3 (580.4–698.1)
$t\frac{1}{2}$ (h)	0.92 0.97 (0.85–1.09)	0.95 0.95 (0.78–1.12)	0.99 1.05 (0.85–1.09)	0.91 1.04 (0.69–1.39)
Kel (h^{-1})	0.76 0.72 (0.64–0.80)	0.75 0.73 (0.60–0.90)	0.71 0.71 (0.51–0.91)	0.71 0.71 (0.54–0.88)
$AUC^{0-\infty}$ 1′*S*,2*R*/1′*S*,2*S*	1.01 1.02 (0.92–1.11)			
$AUC^{0-\infty}$ 1′*R*,2*R*/1′*R*,2*S*	0.94 0.96 (0.88–1.04)			
$AUC^{0-\infty}$ 1′*R*/1′*S*	10.90 11.51 (8.39–14.62)			
$AUC^{0-\infty}$ 2*S*/2*R*	1.05 1.04 (0.97–1.11)			

Median, mean and (CI 95%), $n = 6$ for each time. $p < 0.05$ Kruskal–Wallis test.
[a] 1′*S*,2*R* vs. 1′*R*,2*R*.
[b] 1*S*,2*R* vs. 1′*R*,2*S*.
[c] 1′*S*,2*S* vs. 1′*R*,2*R*.
[d] 1′*S*,2*S* vs. 1′*R*,2*S*.

1′*R*/1′*S* ratio = 11.51 ± 1.2). These results agree with those reported by Murthy et al. [6], who, in an assay on rat hepatic microsomes, obtained 1′*R*/1′*S* ratios higher than 12. Statistically significant differences in the α-hydroxylated compounds were also detected for the C_{max} values between 1′*S* and 1′*R* compounds (Table 5). α-Hydroxylation is the second most important pathway in quantitative terms, with an $AUC^{0-\infty}$ α-OHM/MET ratio of approximately 8.

In conclusion, we developed and validated a HPLC method suitable to study the stereoselective metabolism of MET in vivo. We also report the complete enantioselective study of the metabolism of MET in rats.

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