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Stereoselective Analysis of Carvedilol in Human Plasma and Urine using HPLC after Chiral Derivatization

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ABSTRACT: An enantioselective high-performance liquid chromatographic method for the analysis of carvedilol in plasma and urine was developed and validated using (-)-menthyl chloroformate (MCF) as a derivatizing reagent. Chloroform was used for extraction, and analysis was performed by HPLC on a C18 column with a fluorescence detector. The quantitation limit was 0.25 ng/ml for S(−)-carvedilol in plasma and 0.5 ng/ml for R(+)-carvedilol in plasma and for both enantiomers in urine. The method was applied to the study of enantioselectivity in the pharmacokinetics of carvedilol administered in a multiple dose regimen (25 mg/12 h) to a hypertensive elderly female patient. The data obtained demonstrated highest plasma levels for the R(+)-carvedilol (AUC^{SS}_{0-12} 75.64 vs 37.29 ng/ml). The enantiomeric ratio R(+)/S(−) was 2.03 for plasma and 1.49 for urine (Ae_{0-12} 17.4 vs 11.7 μg). Copyright © 2008 John Wiley & Sons, Ltd.

Key words: carvedilol; enantiomers; HPLC; pharmacokinetics

Background

Carvedilol, (±)-1-(carvazol-4-iloxi)-3-[methoxyphenoxy)-ethyl-amino]-2-propanol (Figure 1), a potent competitive antagonist of β_1, β_2 and α_1 adrenergic receptors, is usually administered as an S-(−) and R-(+) enantiomers mixture in the treatment of hypertension, ischaemic heart disease and congestive heart failure [1–3]. The carvedilol enantiomers exhibit different pharmacological effects, the blockade of the β_1 adrenergic receptor being primarily attributed to the S(−)-carvedilol, whereas the two enantiomers are considered to be equipotent with respect to the blockade of the α_1 adrenergic receptor [3]. The kinetic disposition of carvedilol administered to healthy volunteers, hypertensive patients and hypertensive patients with renal insufficiency is enantioselective, with higher plasma concentrations of the R(+)-carvedilol [4–8]. The oral bioavailability of R(+)-carvedilol is also about 50% higher than S(−)-carvedilol in healthy volunteers, with an indication of significant enantioselectivity in the first passage effect [9,10]. The binding to plasma proteins is also higher for R(+)-carvedilol [3].

The investigation of enantioselectivity in the kinetic disposition of carvedilol requires the availability of a method for the analysis of individual isomers with sensitivity and accuracy compatible with the low plasma or urine concentrations (ng/ml) observed after the administration of single or multiple doses of the antihypertensive drug. In this regard, the enantioselective analysis of carvedilol in animal or human plasma has been obtained by direct HPLC methods using a chiral stationary phase

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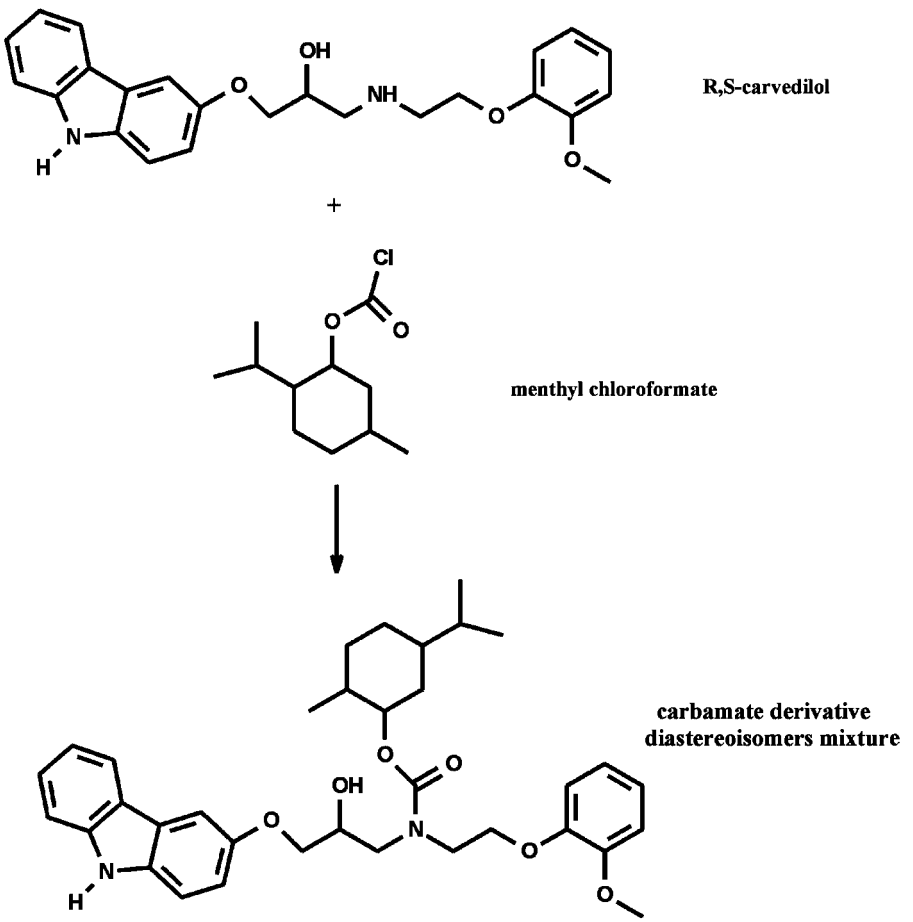

Figure 1. Scheme of derivatization of carvedilol with (-)-menthyl chloroformate (MCF)

column [11] and by derivatization using enantiomerically pure reagents [12,13]. Hence, Eisenberg *et al*. [12] used the 2,3,4,6–tetra-O-acetyl-β-D-glycopyranosyl isothiocyanate reagent for the chiral derivatization of carvedilol, in a reaction selectively carried out over a period of 30 min at room temperature. The same derivatization procedure was used by other authors such as Neugebauer *et al*. [6] in the investigation of stereoselectivity in presystemic carvedilol metabolism in young healthy volunteers, Neugebauer *et al*. [7] in the investigation of the kinetic disposition of carvedilol in patients with cirrhosis and Zhou and Wood [8] in the investigation of the influence of the oxidative phenotype of CYP2D6 in the stereoselective kinetic disposition of carvedilol. The derivatization with S-naproxen reported by Spahn *et al*. [13] requires 1 h at 50°C and was used for the evaluation of stereoselectivity in the pharmacokinetics of carvedilol administered p.o. and i.v. to healthy volunteers. Stahl *et al*. [14] used R(+)-phenylethyl isocyanate as the derivatization reagent in a more rapid reaction requiring approximately 30 min. The method was used for the evaluation of enantioselectivity in the kinetic disposition of carvedilol administered to rats in the form of a racemic mixture.

Menthyl (-)-chloroformate (MCF) has been used for the conversion of β-blocker enantiomers such as metoprolol, atenolol, pindolol, acebutolol, bisoprolol and celiprolol, among others, to diastereoisomeric derivatives which are separated on standard reverse-phase columns [15–18] or gas chromatography [19]. Chloroformate reacts with amines and alcohols, respectively

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producing carbamate and carbonated derivatives [15,19]. Mevhar [15] reported that, in the presence of water, the reaction occurs only with the amino group, thus forming carbamate derivatives which can be extracted from the aqueous phase with an organic solvent at acid or basic pH. Kim *et al*. [20] reported that the procedure of metoprolol derivatization with this chiral reagent does not result in racemization.

The present study developed a rapid and sensitive method for the enantioselective analysis of carvedilol in plasma and urine using the formation of diastereoisomers with MCF, a reagent not previously used for chiral carvedilol derivatization. The method was applied to the investigation of enantioselectivity in the kinetic disposition of carvedilol in an elderly hypertensive female patient.

Experimental

Chemicals

Racemic carvedilol was obtained from Baldacci Laboratories S/A (São Paulo, Brazil) and propranolol (used as internal standard) was obtained from Novartis Bioscience S/A (São Paulo, Brazil). The enantiomerically pure chiral agent MCF was obtained from Sigma (St Louis, MO, USA) and the HPLC grade solvents used in the extraction procedure and for the mobile phase of the chromatographic system were obtained from Merck (Darmstadt, Germany). Oral tablets containing 25 mg of racemic carvedilol (Divelol®) were obtained from Baldacci Laboratories S/A (Sao Paulo, Brazil).

Instruments and chromatographic conditions

A Shimadzu (Kyoto, Japan) chromatographic system equipped with a Rheodyne manual injector with a 20 µl sampler, an isocratic elution pump model LC–10AS, a fluorescence detector model RF 10AXL, operating at 285 nm (λ ex) and 680 nm (λ em), and an integrator model C-R6A, was used.

The carvedilol diastereoisomers were separated on a Lichrospher® 100 RP8 column (125 × 4 mm i.d., 5 µm particles) with a similar 4 × 4 mm pre-column and with a mobile phase consisting of a mixture of 0.25 N acetate buffer (pH 3): methanol (27:73 v/v) at a flow rate of 1 ml/min.

Standard solutions

The stock solution of racemic carvedilol was prepared in methanol at a concentration of 1 mg/ml. This solution was then used to prepare solutions at concentrations of 0.01, 0.02, 0.04, 0.10, 0.20, 0.40, 0.60, 1.00, 2.00, 4.00 and 8.00 µg/ml racemic carvedilol/ml methanol. Propranolol at a concentration of 12 µg/ml methanol was used as the internal standard.

The MCF solution was prepared daily in dichloromethane at 2% concentration (v/v). An aqueous solution of 0.1 M sodium hydroxide was also prepared. Water was obtained by double distillation and purified additionally with a Milli-Q system (Millipore Corporation, MA, USA).

Sample preparation

Plasma or urine samples (1 ml) were spiked with 0.3 µg propranolol (internal standard) and 50 µl of an aqueous solution of 0.1 M sodium hydroxide and extracted with 6 ml chloroform for 30 min in a horizontal shaker at 200 ± 10 cycles/min. After centrifugation at 1800 × *g* for 4 min, the organic phases (5 ml) were transferred to conical tubes and evaporated to dryness under an air flow at room temperature. The residues obtained by liquid–liquid extraction were dissolved in 200 µl of an aqueous solution of 0.1 M sodium hydroxide and 200 µl of the chiral reagent MCF at 2% concentration in dichloromethane (v/v) was added and the mixture was shaken in a vortex for 2 min. After the addition of 1 ml of water, the carvedilol diastereoisomers were extracted with 3 ml chloroform for 2 min in a vortex shaker. The samples were centrifuged at 1800 × *g* for 4 min and the organic phases (2.5 ml) were transferred to conical 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 HPLC system.

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Plasma and urine standard curve and linearity

The human plasma and urine pools used for the validation of the analytical method were first tested for the determination of peaks interfering with those of the carvedilol enantiomers or the internal standard.

The calibration curves were constructed from 1 ml samples of blank plasma or urine spiked with 25 µl of each diluted carvedilol solution (0.25–200 ng/ml) supplemented with the internal standard solution and extracted as described previously. The linearity was studied by analysis of plasma and urine samples spiked with increasing carvedilol concentrations in relation to those employed for the construction of the calibration curves. The method was considered to be linear up to the highest concentration analysed with a coefficient of variation $<15\%$. Plasma and urine samples containing concentrations of 0.25 to 200 ng/ml of each enantiomer were analysed in triplicate.

Limit of quantification (LOQ) and recovery

The quantification limit was determined as the lowest intra-assay concentration analysed (n=5) with a coefficient of variation of less than 20%. Samples containing concentrations of 0.25 and 0.5 ng/ml of each enantiomer were analysed in five replicates.

The relative analytical recovery of the carvedilol enantiomers from plasma and urine was determined by comparison of the ratios of the peak areas obtained after extraction from plasma or urine to the ratios of the peak areas obtained after the procedure of direct derivatization of the standard solutions. This study was carried out by analysing two concentrations of the enantiomers (2.5 and 50 ng/ml) in plasma and urine using four replicates.

Precision and accuracy

The precision was assessed by determining the intra- (n=10) and inter-assays (n=5) coefficients of variation of the analysis of spiked blank plasma and urine (1 and 25 ng of each enantiomer per ml). Accuracy (% systematic error) was obtained by determining the agreement between the results obtained experimentally and the real values of the enantiomers in the sample.

Selectivity

The interference of other possibly co-administered drugs was determined by injecting standard solutions after the procedure of derivatization, at concentrations similar to those observed at the therapeutic doses. The following drugs were tested: acetylsalicylic acid, atenolol, carbamazepine, diazepan, sotalol, pindolol, amiodarone, hydrochlorothiazide, amitryptiline, chlorpromazine, nitrazepan and captopril.

Biological samples

A hypertensive patient (66-year-old woman, 67 kg) was included in the investigation after giving written informed consent to participate. The patient received multiple p.o. doses of 25 mg racemic carvedilol every 12 h (Divelol®).

Five ml blood samples were collected at 0 and 30 min and 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after administration, using heparin (Liquemine® 5000 IU, Roche) as anticoagulant. The blood samples were centrifuged at $1800 \times g$ for 10 min and the plasma was stored at -20°C until the time for chromatographic analysis.

Urine was collected over intervals of 0–4, 4–8 and 8–12 h after carvedilol administration. The total volume of urine was recorded and the samples were stored at -20°C until the time for chromatographic analysis.

Pharmacokinetic analysis

The kinetic disposition of the enantiomers S(−) and R(+)-carvedilol was determined in the state of equilibrium in the dose interval (0–12 h) of carvedilol administration based on the open bicompartmental model.

The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. The elimination half-life ($t_{1/2}\beta$) was determined directly by the graphic method ($\log c$ vs t). The rate constants Ka, α and β were calculated by the equation $0.693/t_{1/2a}$, $0.693/t_{1/2\alpha}$ and $0.693/t_{1/2\beta}$, respectively. The areas under the plasma concentration versus time curves (AUC^{ss}_{0-12}) were calculated by

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the trapezoid method. This parameter was used for the calculation of the total apparent clearance (Cl/f=dose/AUC^{ss}_{0-12}) and of the apparent distribution volume ($Vd/f=Cl/f/\beta$). The mean absorption time (*MAT*) was calculated by the equation $MAT=1/Ka$.

The renal clearance (CL_R) of carvedilol was calculated by the equation $Ae^{SS}_{0-12}/AUC^{SS}_{0-12}$, where *Ae* is the amount of carvedilol excreted in the unaltered form during the dose interval (0–12 h). For the calculation of *Ae*, urinary concentration (*Cu*) was multiplied by the urine volume obtained during the collection interval (*Vu*). The total amount excreted into urine was calculated by the sum of the values for each interval.

The fraction of the carvedilol dose excreted in the unaltered form (*Fel/F*) was calculated by the equation $Fel/F=Ae^{SS}_{0-12}$/dose.

Results and Discussion

In the present investigation, MCF was used for derivatization of the carvedilol isomers in alkaline medium. The diastereoisomeric derivatives (Figure 1) were separated on a C18 reverse-phase column and detected by fluorescence (λ_{exc} 285 nm; λ_{em} 680 nm) (Figure 2). The reaction occurred quickly (within approximately 2 min) and the diastereoisomeric derivatives were eluted from the reverse-phase column within less than 25 min. In agreement with previous studies reporting higher plasma concentrations of the R(+) enantiomer after p.o. administration of racemic carvedilol to healthy volunteers or to patients with different diseases, the sequence of elution was S(−) and R(+) [6,8,13].

The blank plasma and urine pools used for the validation of the method were analysed previously and did not present peaks at the time of retention of the carvedilol enantiomers or of the internal standard (Figures 2 and 3). The calibration curves for both enantiomers in plasma and urine were constructed in the interval from 0.25 to 200 ng/ml, with correlation coefficients higher than 0.99. The carvedilol isomers were extracted from plasma with chloroform in alkaline medium, with more than 93% recovery rates (Table 1)

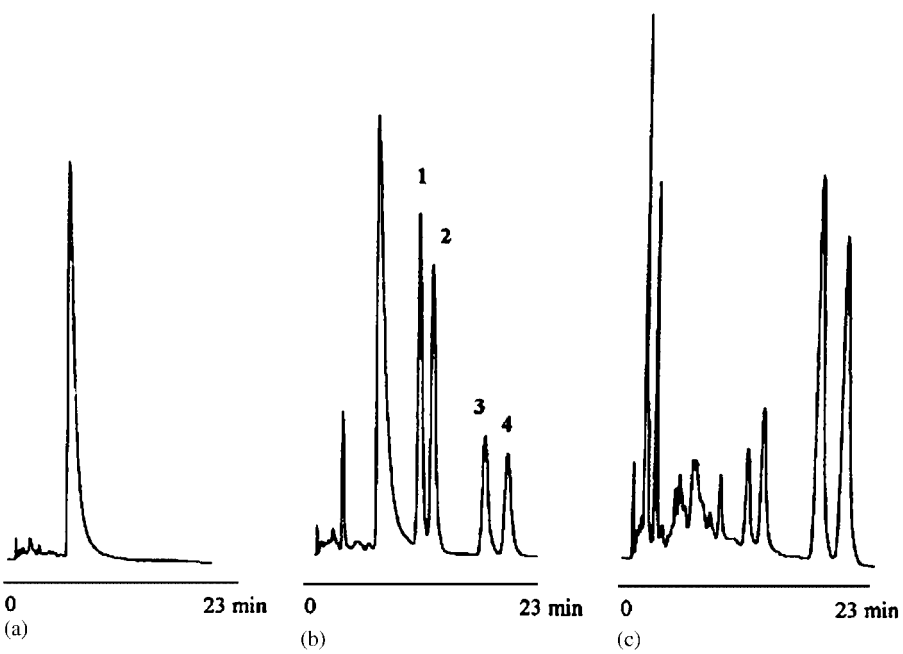

Figure 2. The HPLC chromatograms resulting from the derivatization with MCF of (a) blank human plasma; (b) human plasma spiked with 30 ng/ml of the racemic drug (R,S)-carvedilol and (R,S)-propranolol (internal standard); peaks **1** and **2** correspond to S(−) and R(+)-carvedilol, respectively; peaks **3** and **4** correspond to the propranolol enantiomers (IS) and (c) plasma sample of a patient treated with 25 mg of racemic carvedilol/12 h

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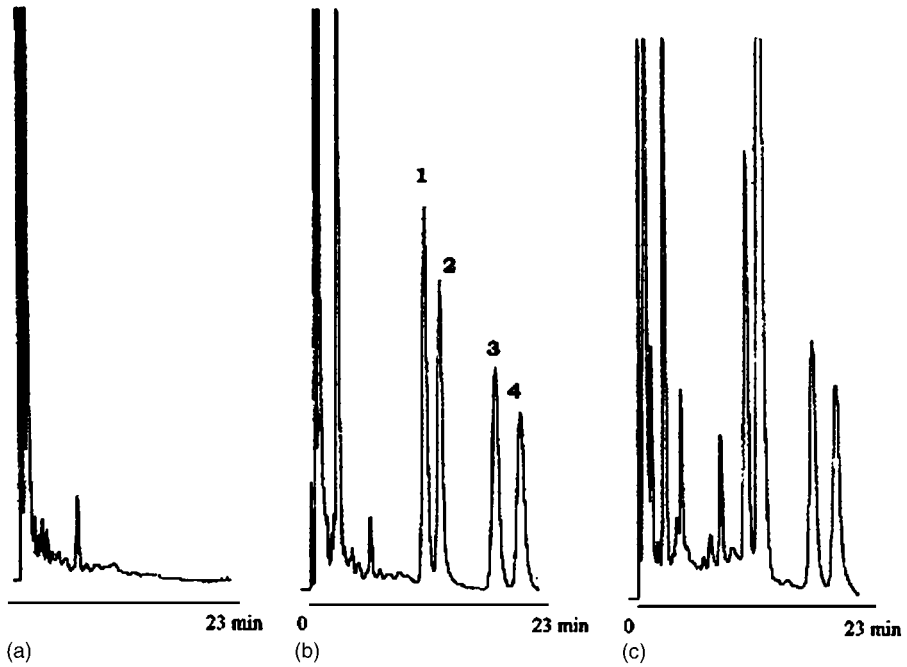

Figure 3. The HPLC chromatograms resulting from the derivatization with MCF of (a) blank human urine; (b) human urine spiked with 30 ng/ml of the racemic drug (R,S)-carvedilol and (R,S)-propranolol (internal standard); peaks **1** and **2** correspond to S(−) and R(+)-carvedilol, respectively; peaks **3** and **4** correspond to the propranolol enantiomers (IS) and (c) urine sample of a patient treated with 25 mg of racemic carvedilol/12 h

Table 1. Confidence limits for the analysis of carvedilol enantiomers in plasma and urine

	Plasma	Urine
Recovery %		
S(−)-carvedilol	99.3	98.5
R(+)-carvedilol	93.8	99.8
Quantitation limit ng/ml (CV%) n=5		
S(−)-carvedilol	0.25 (12)	0.5 (4.5)
R(+)-carvedilol	0.5 (7.9)	0.5 (7.2)
Linearity ng/ml (r^2)		
S(−)-carvedilol	0.25–200 (0.9996)	0.5–200 (0.9991)
R(+)-carvedilol	0.5–200 (0.9882)	0.5–200 (0.9968)

CV, coefficient of variation.

regardless of the concentrations analysed. Eisenberg *et al*. [12], using solid phase extraction, also obtained high values for carvedilol recoveries. In the present study, linearity was observed up to the highest experimental concentration, i.e. 200 ng of each enantiomer/ml of plasma or urine (Table 1). The quantification limit for the analysis of S(−)-carvedilol in plasma was defined as 0.25 ng/ml, whereas the quantification limit for R(+)-carvedilol in plasma and for both enantiomers in urine was defined as 0.5 ng/ml (Table 2). These results permit us to infer that the present method is as sensitive as the techniques that use a direct method with a chiral stationary phase column [11] or indirect methods employing enantiomerically pure reagents such as S-naproxen hydrochloride [13] (1 ng/ml), or 2.3,4,6–tetra-O-acetyl-β-D-glycopyranosyl [4,13] (1 ng/ml and 2 ng/ml, respectively). The precision, with coefficients of variation of less than 10%, and the

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Table 2. Precision and accuracy for the analysis of carvedilol enantiomers in plasma and urine

	Plasma		Urine	
	Precision (CV%)	Accuracy (%SE)	Precision (CV%)	Accuracy (%SE)
S(−)-carvedilol				
Intra-assay (n=10)				
1 ng/ml	8.6	6.4	7.1	−0.7
25 ng/ml	8.4	−5.3	9.7	−8.4
Inter-assay (n=5)				
1 ng/ml	9.1	5.4	6.0	−1.4
25 ng/ml	8.0	−4.6	7.7	−2.1
R(+)-carvedilol				
Intra-assay (n=10)				
1 ng/ml	7.2	0.9	7.9	−10.2
25 ng/ml	8.6	5.1	6.5	−8.8
Inter-assay (n=5)				
1 ng/ml	8.3	1.5	8.0	−2.3
25 ng/ml	7.8	2.6	6.6	−4.0

CV, coefficient of variation; SE, systematic error.

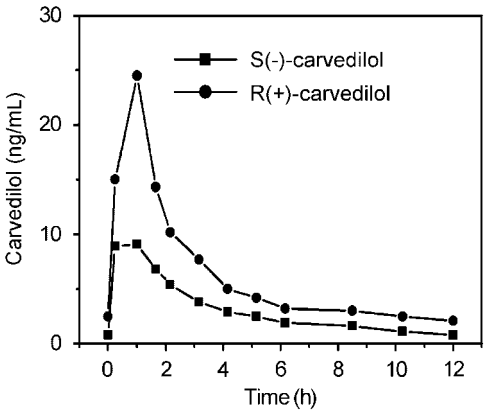

Figure 4. Plasma concentration versus time curves of S(−) and R(+)-carvedilol after oral administration of 25 mg of racemic carvedilol/12 h to an elderly hypertensive patient

accuracy, with systematic error percentages lower than 10% (Tables 1 and 2) for both carvedilol isomers, do not suggest racemization and fulfil the most rigid criteria for the validation of analytical methods to be applied to studies of kinetic disposition. The results of the selectivity study permitted us to infer that the method is highly specific as a function of the derivatization reaction and of fluorescence detection. The following drugs were not detected: acetylsalicylic acid, atenolol, carbamazepine, diazepan, sotalol, pindolol, amiodarone, hydrochlorothiazide, amitryptiline, chlorpromazine, nitrazepan and captopril.

The analytical method developed and validated was applied to the investigation of stereoselectivity in the kinetic disposition of carvedilol administered p.o. in the racemic form in a multiple-dose regimen to an elderly hypertensive female patient. The plasma concentration versus time curves obtained in the investigation of the hypertensive elderly patient studied here (Figure 4) were used to calculate the pharmacokinetic parameters listed in Table 3. The results obtained permitted us to infer the plasma accumulation of R(+)-carvedilol ($AUC_{R(+)/S(-)}$ ratio of 2.03) was the consequence of its lower clearance and smaller apparent distribution volume. In agreement, Neugebauer *et al*. [6] reported $AUC_{R(+)/S(-)}$ ratios of 1.6 to 4.4 after p.o. administration of a single 50 mg dose of racemic carvedilol to 10 healthy volunteers. Spahn *et al*. [13] reported $AUC_{R(+)/S(-)}$ ratio of 3.3 in the investigation of the same population (n=3). The data reported by Neugebauer *et al*. [7] showed $AUC_{R(+)/S(-)}$ ratios of 1.6 after p.o. administration of 25 mg racemic carvedilol to six patients with cirrhosis of the liver. Tenero *et al*. [21] showed that the $AUC_{R(+)/S(-)}$ ratios were not changed as a function of congestive heart failure and found values of 1.29 to 4.04 in the investigation of patients treated with p.o. doses of 6.25 to 50 mg of racemic carvedilol.

The renal carvedilol excretion is of low significance, with data reported in the literature showing values of less than 2% of the dose administered. The data concerning renal carvedilol excretion obtained in the present study show that renal clearance practically did not differ between the enantiomers (0.164 for S(−)-carvedilol vs 0.162 for R(+)-carvedilol) and that the amount of the enantiomers excreted into urine (*Ae*) reflects plasma data, with an $Ae_{R(+)/S(-)}$ ratio of 1.5.

In conclusion, the method developed and validated in the present study shows sensitivity, selectivity and precision for the investigation of enantioselectivity in the pharmacokinetics of carvedilol administered in a p.o. multiple dose regimen. It is also important to point out that the time needed for the derivatization reaction with MCF is much shorter than those reported

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Table 3. Pharmacokinetics parameters of carvedilol enantiomers following oral doses of 25 mg racemic carvedilol/12h to an elderly hypertensive patient

	R(+)-carvedilol	S(−)-carvedilol
C_{max} (ng/ml)	24.5	9.1
t_{max} (h)	1.0	1.0
$t^{a}_{1/2}$ (h)	1.54	1.73
Ka (h^{-1})	0.45	0.40
MAT (h)	0.65	0.58
AUC^{SS}_{0-12} (ng.h/ml)	75.64	37.29
$t_{1/2}\alpha$ (h)	0.75	0.5
α (h^{-1})	0.93	1.39
Vd/f (l)	1271.2	2793.4
Cl/f (l/h)	165.0	335.2
$t_{1/2}\ \beta$ (h)	5.2	5.8
β (h^{-1})	0.13	0.12
Cl_R (l/h)	0.16	0.16
Ae (μg)	17.4	11.7
Fel/f (%)	0.14	0.09
AUC^{SS}_{0-12} R(+)/S(−)	2.03	

for indirect methods using other chiral agents and it is recommended when the clinical study involves a large number of plasma or urine samples.

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