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Journal of Chromatography B, 843 (2006) 73–77

JOURNAL OF CHROMATOGRAPHY B

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Enantioselective and highly sensitive determination of carvedilol in human plasma and whole blood after administration of the racemate using normal-phase high-performance liquid chromatography

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Received 9 August 2005; accepted 21 May 2006
Available online 19 June 2006

Abstract

A highly sensitive HPLC method for enantioselective determination of carvedilol in human whole blood and plasma was developed. Carvedilol and *S*-carazolol as an internal standard extracted from whole blood or plasma were separated using an enantioselective separation column (Chiralpak AD column; 2.0 Ø × 250 mm) without any chiral derivatizations. The mobile phase was hexane:isopropanol:diethylamine (78:22:1, v/v). The excitation and emission wavelengths were set at 284 and 343 nm, respectively. The limits of quantification for the *S*(−)- and *R*(+)-carvedilol enantiomers in plasma and blood were both 0.5 ng/ml. Intra- and inter-day variations were less than 5.9%. As an application of the assay, concentrations of carvedilol enantiomer in plasma and blood samples from 15 patients treated with carvedilol for congestive heart failure were determined.
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Keywords: Carvedilol enantiomers; HPLC; Human whole blood; Human plasma

1. Introduction

Carvedilol, 1-(carbazol-4-yloxy-3-[[2-(*O*-methoxyphenoxy) ethyl]amino]-2-propranol, is a drug with β- and α_1- receptor blocking activity approved for the treatment of congestive heart failure (CHF). However, as tolerance to treatment with carvedilol varies widely among individuals, careful titration and treatment initiation are necessary in daily practice. Carvedilol is administered clinically as a racemic mixture of the *R*(+)- and the *S*(−)-enantiomer. Each enantiomer exhibits different pharmacological effects, i.e., the β-receptor blocking activity of the *S*-enantiomer is about 200 times higher than that of *R*-carvedilol, whereas both enantiomers are equipotent α-blockers [1,2]. Each enantiomer exhibits different pharmacokinetic behavior in human subjects [3–6]. Therefore, it is very important to measure the blood or plasma concentrations of each carvedilol enantiomer in order to clarify the pharamacokinetic and pharamacodynamic characteristics of carvedilol upon clinical application.

HPLC methods using chiral derivatization have been developed and applied for simultaneous determination of each enantiomer of carvedilol in human plasma with a quantification limit of 0.5–1.6 ng/ml [7–9]. However, these methods require a derivatization agent, or using a specialized electrophoretic method. We tried to develop a more sensitive and simple method with small volume of plasma or blood samples. In this paper, we describe a new highly sensitive method for carvedilol determination in whole blood and plasma in which each enantiomer is separated by a chiral column without any chiral derivatization.

2. Experimental

2.1. Chemicals and materials

Racemic carvedilol was obtained from Daiichi Pharma Ltd., Japan (Fig. 1A), and was used as a certified reference com-

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doi:10.1016/j.jchromb.2006.05.018

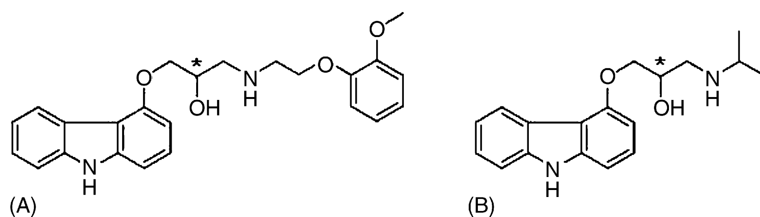

Fig. 1. Chemical structure of carvedilol (A) and carazolol (B). The asterisks indicate the asymmetrical carbon atoms.

pound for quantitative analysis. Carazolol (Wako Ltd., Japan) (Fig. 1B) was used as an internal standard. Diethylether, hexane, isopropanol and diethylamine were purchased from Nakarai Tesque Inc., Japan. Blank whole blood and plasma were obtained from fasted healthy volunteers.

2.2. Preparation of reagents and stock solutions

Authentic racemic carvedilol was dissolved in isopropanol to a concentration of 1 mg/ml as a stock solution. *S*-carazolol as the internal standard was pre-separated from racemic carazolol (100 μg/ml in isopropanol) using an enantio-selective column. The retention time of *S*- and *R*-carazolol was 15 and 40 min in the following HPLC condition, respectively. Therefore, *S*-carazolol was selected as internal standard in this assay method for reducing working time. Separation was achieved with an enantio-selective column (Chiralpak AD column; 4.6 Ø × 250 mm, Daicel Chemical Industries Ltd., Tokyo, Japan). The mobile phase for the enantio-selective separation of carazolol was hexane:isopropanol:diethylamine (70:30:0.1, v/v), and the flow rate was 1.0 ml/min. The excitation and emission wavelengths were set at 284 and 343 nm, respectively. The temperature of the column oven was set at 25 °C. The eluted *S*-carazolol solution was collected, and evaporated under a nitrogen gas (99.5%) stream. *S*-carazolol (1 μg/ml in isopropanol as a stock solution) was prepared. Various carvedilol working solutions with concentrations between 0.5 and 100 ng/ml were prepared by mixing a carvedilol stock solution and a internal standard stock solution, and diluting with the mobile phase (hexane:isopropanol:diethylamine = 78:22:1, v/v) for HPLC analysis. Also, the stock solution of internal standard was diluted with the mobile phase to obtain the working internal standard solution of 100 ng/ml. All stock solutions and working solutions were stored at 4 °C.

2.3. Sample preparation

Carvedilol in plasma was determined as follows: 100 μl of Kolthoff-buffer (pH 8.0, 0.1 M KH_2PO_4, 0.05 M $Na_2B_4O_7$) and 50 μl of internal standard solution were added to 50 μl of plasma. After addition of 6 ml of diethylether, the tube was capped and mechanically shaken for 20 min, then centrifuged for 5 min at 3000 × *g*. Five milliliters of the organic layer was transferred to another tube and evaporated to dryness under a nitrogen gas (99.5%) stream. The residue in the tube was reconstituted with 100 μl of mobile phase, and 50 μl of the solution was injected onto the HPLC column.

Carvedilol in whole blood was determined as follows: A blood sample was prepared by adding 100 μl of distilled water, 50 μl of internal standard solution and 100 μl of Kolthoff buffer

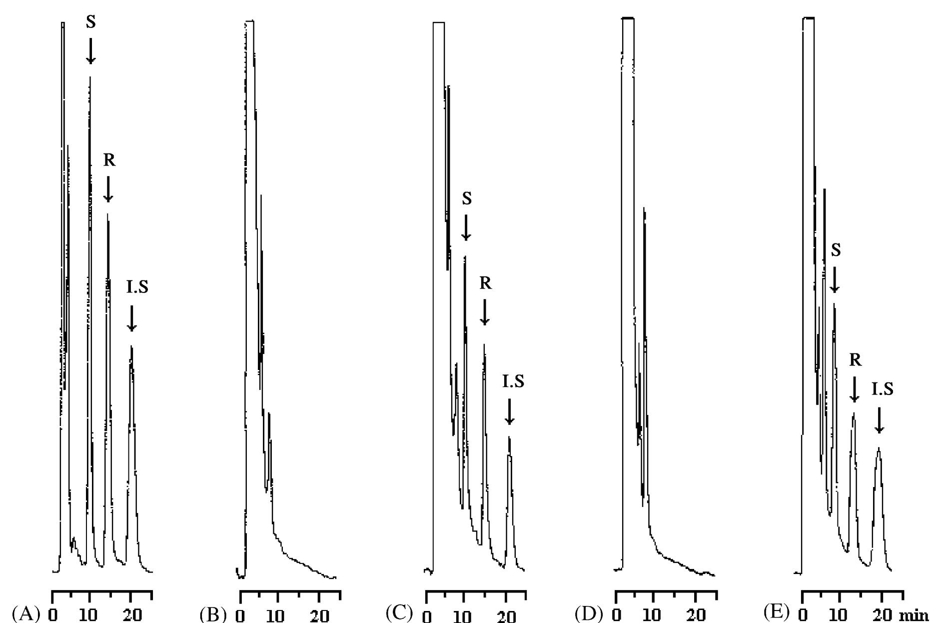

Fig. 2. HPLC chromatogram of *R*(+)- and *S*(−)-carvedilol. (A) Standard solution (2.5 ng/ml of each enantiomers), (B) drug-free blank human plasma, (C) spiked plasma (2.5 ng/ml of each enantiomers in human plasma), (D) drug-free blank human blood, (E) spiked blood (2.5 ng/ml of each enantiomers in human blood).

Table 1
Intra and Inter-day precision and accuracy of the HPLC assay for $S(-)$- and $R(+)$-carvedilol

Enantiomer concentration of carvedilol (ng/ml)	$S(-)$-carvedilol				$R(+)$-carvedilol			
	Intra-day ($n=5$)		Inter-day ($n=5$)		Intra-day ($n=5$)		Inter-day ($n=5$)	
	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)
Plasma (0.508–25.15)	2.2–3.4	95.1–107.1	0.5–5.9	98.5–100.6	2.2–6.8	94.5–109.1	0.5–6.6	98.4–100.3
Blood (0.522–26.08)	2.4–4.8	97.5–103.6	0.1–4.8	99.2–102.1	3.3–5.5	98.3–102.1	0.1–5.8	98.4–100.6

to 50 μl of blood. After addition of 6 ml of diethylether to the sample, the carvedilol concentration was determined using the same method as that for plasma described above.

2.4. Instrumentation

The HPLC system used for the assay consisted of a liquid chromatograph (Shimadzu LC-10ADVP) equipped with a wavelength fluorescence detector (RF-10AXL, Shimadzu), a data processor (C-R8A, Shimadzu), and a column oven (CTO-2A, Shimadzu). Separation was achieved with an enantioselective column (Chiralpak AD column; 2.0 Ø × 250 mm, Daicel Chemical Industries Ltd., Tokyo, Japan). The mobile phase was hexane:isopropanol:diethylamine (78:22:1, v/v), and the flow rate was 0.3 ml/min. The excitation and emission wavelengths were set at 284 and 343 nm, respectively. The temperature of the column oven was set at 45 °C. The limits of detection for $S(-)$- and $R(+)$-carvedilol (based on a signal-to-noise ratio not less than 3:1) were approximately 3 and 4.5 pg in this chromatographic system, respectively.

2.5. Calibration

The standard carvedilol samples in plasma or blood were prepared just before these samples were extracted. Fifty microliters of carvedilol working solution were added to 50 μl of plasma or blood, and then extraction of standards was carried out as described procedure. The slope and intercept of the calibration curves for each carvedilol enantiomer were obtained by linear regression of the peak height ratios of each carvedilol enantiomer to the internal standard versus the concentration of each carvedilol enantiomer within the range 0.5–25 ng/ml.

2.6. Validation

For assessing the precision and accuracy of carvedilol measurements in plasma or blood, five samples of carvedilol dissolved in blank plasma, blank blood (0.5, 2.5, 5, 25 ng/ml each enantiomer) were analyzed within 1 day for assessing intra-day variability, and among 5 days for assessing inter-day variability. The limit of quantification which were less than 10% coefficient of variation using the assay method was evaluated. A total absence of matrix interference was confirmed by analysis of blank plasma or blank blood. The recoveries of carvedilol were determined by calculating peak height ratio of carvedilol for the extracted samples and non-extracted standards.

2.7. Application

The carvedilol concentrations in both plasma and blood were determined for a 55-year-old male patient treated with carvedilol due to dilated cardiomyopathy. Blood samples were drawn at four points after the dose during 1 day at steady state after repeated oral administration of 15 mg carvedilol once a day. In this study, the carvedilol enantiomer concentrations at various doses were also determined in plasma samples taken from 14 CHF patients (age: 67 ± 8 year (mean ± S.D.), sex: men 12, women 2) treated with carvedilol. Blood samples were drawn at 3–4 points per patient during 1 day at steady state after repeated oral administration of 2.5–20 mg carvedilol 2 divided doses. The blood samples were centrifuged 2000 × *g*, for 10 min at room temperature and the obtained plasma was stored at −20 °C until analysis. This protocol had been approved by Sakakibara Heart Institute, and the patients gave their informed consents to participate.

3. Results and discussion

3.1. Chromatography

The retention times of $S(-)$- and $R(+)$-carvedilol and the internal standard (I.S.) were 11, 15, and 23 min, respectively (Fig. 2). No other peaks corresponding to these retention times

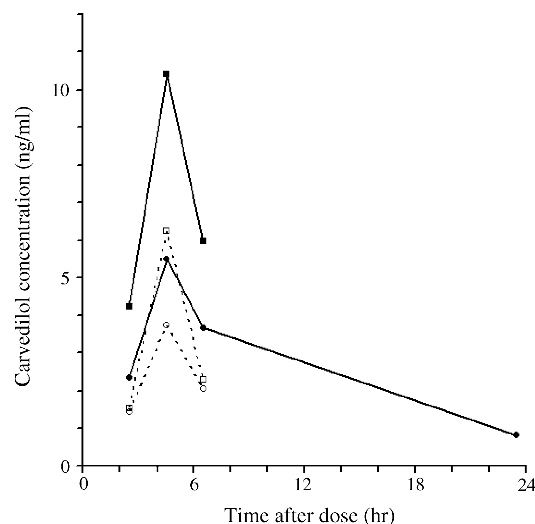

Fig. 3. Plasma and blood concentration–time profiles of *S*-carvedilol (●: plasma; ○: blood) and *R*-carvedilol (■: plasma; □: blood) in a CHF patient after oral administration of 15 mg carvedilol.

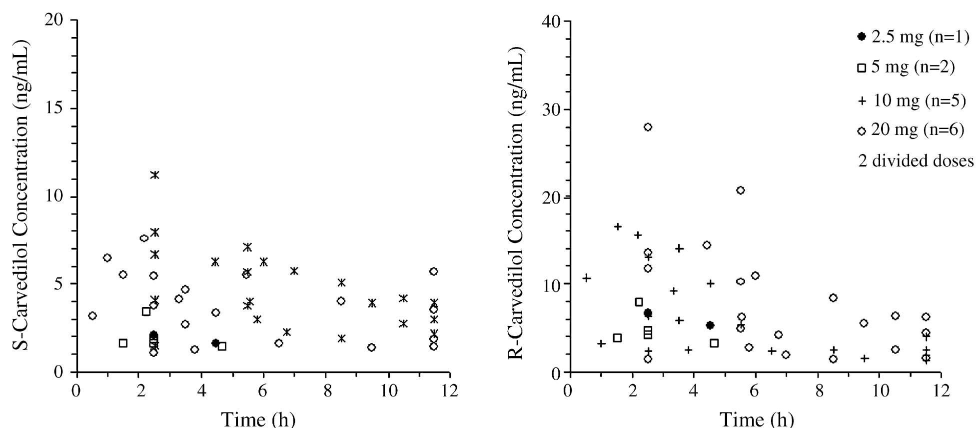

Fig. 4. Plasma concentration–time profiles of *S*-carvedilol and *R*-carvedilol in 14 CHF patients after oral administration of 2.5–20 mg carvedilol (●: 2.5 mg; □: 5 mg; +: 10 mg; ◯: 20 mg) in two divided doses.

were noted on the chromatograms for drug-free blank human plasma and blood.

3.2. Calibration

The standard curve was prepared by analyzing the plasma and blood solutions containing 1, 5, 10, 50 ng/ml racemic carvedilol. Excellent linearity was noted between the concentration of carvedilol and the peak height ratio of carvedilol to the I.S. within the range 1–50 ng/ml. The coefficient of correlation (r) was 0.999 and the regression equation passed the original point.

3.3. Validation

Intra- and inter-assay variabilities were determined at five concentrations of each carvedilol enantiomer in plasma and blood within the range 0.5–25 ng/ml (Table 1).

The observed mean concentrations of carvedilol ranged from 95 to 109% of the spiked concentrations and the CV values ranged from 2.2 to 6.8% in the repeated intra-day assay, irrespective of the concentrations of carvedilol enantiomers and whether the samples were dissolved in blood or plasma. The observed mean concentrations of carvedilol ranged from 97 to 102% of the spiked concentrations and the CV values ranged from 0.1 to 6.6% in the repeated inter-day assay, irrespective of the concentrations of carvedilol enantiomers and whether the samples were dissolved in blood or plasma. The limits of quantification for the *S*(−)- and *R*(+)-enantiomers in plasma and blood were both 0.5 ng/ml. The recoveries of *S*(−)- and *R*(+)-carvedilol in plasma were 64.8% (CV = 4.0%) and 67.6% (CV = 3.8%) at 5 ng/ml, respectively. The recoveries of *S*(−)- and *R*(+)-carvedilol in blood were 75.6% (CV = 4.1%) and 74.8% (CV = 4.3%) at 5 ng/ml, respectively.

3.4. Carvedilol concentration in plasma and whole blood of the CHF patients

The method developed in this study was applied for determining the concentrations of carvedilol enantiomers in plasma or whole blood of 15 CHF patients. The plasma and blood concentration–time profiles of *S*(−)- and *R*(+)-carvedilol in a patient are shown in Fig. 3. *S*(−)- and *R*(+)-carvedilol were determined at 2.5, 4.5, 6.5 and 23.5 h after oral administration of 15 mg of racemic carvedilol in this patient. Maximum plasma concentrations of *S*(−)- and *R*(+)-carvedilol were 5.49 and 10.41 ng/ml, respectively, and the corresponding trough concentrations were 0.82 and 0.33 ng/ml (below the lower limit of concentration), respectively. The average blood/plasma concentration ratio was 0.6 in this study, which is similar to the value reported by Fujimaki et al. [4].

For 14 CHF patients treated with 2.5–20 mg carvedilol in two divided doses, the plasma concentrations–time profiles are presented in Fig. 4. Both enantiomer concentrations showed highly interindividual variability even at the same dose. Trough concentrations could be determined after 5 and 10 mg doses per 12 h. In this study, we assayed carvedilol concentrations using small sample volume of 50 μl and the lowest standard carvedilol concentration of 0.5 ng/ml, but more sensitive detection might be obtained when the larger sample volume was used for the assay. This assay method will give a great advantage when we are obliged to assay plasma or blood carvedilol concentration using small volume of biological samples, for example, in pharmacokinetic and pharmacodynamic studies participating neonates and/or young children.

4. Summary

A sensitive HPLC method for enantioselective determination of carvedilol in human whole blood and plasma was developed in this study. The limits of quantification of the *S*(−)- and the *R*(+)-enantiomers were both 0.5 ng/ml in plasma and whole blood. This method was able to determine the concentration of carvedilol in a very small volume (50 μl) of human plasma and blood with good precision and accuracy. The present method will be useful for pharmacokinetic and pharmacodynamic studies of carvedilol enantiomers during steady state condition in patients after carvedilol treatment.

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