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Separation of carvedilol enantiomers in very small volumes of human plasma by capillary electrophoresis with laser-induced fluorescence

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Abstract

A sensitive capillary electrophoretic method for the determination of carvedilol enantiomers in 100 μl of human plasma has been developed and validated. Carvedilol and the internal standard carazolol are isolated from plasma samples by liquid–liquid extraction using diethylether. A sensitive and selective detection is provided by helium–cadmium laser-induced fluorescence. The total analysis time is 17.5 min, about 30 min are needed for the sample preparation. The linearity of the assay ranges from 1.56 to 50 ng/ml per carvedilol enantiomer. The limits of quantification (LOQ) for the carvedilol enantiomers in 100 μl of human plasma are 1.56 ng/ml. The inter-day accuracy for *R*-carvedilol is between 95.8 and 103% (104% at LOQ) and for *S*-carvedilol between 97.1 and 103% (107% at LOQ); the inter-day precision values are between 3.81 and 8.64% (10.9% at LOQ) and between 5.47 and 7.86% (7.91% at LOQ) for *R*- and *S*-carvedilol, respectively. The small sample volume needed is especially advantageous for the application in clinical studies in pediatric patients. As an application of the assay concentration/time profiles of the carvedilol enantiomers in a 5-year-old patient receiving a test dose of 0.09 mg/kg carvedilol are reported.

Keywords: Enantiomer separation; Carvedilol

1. Introduction

Carvedilol, a β-receptor blocker with additional vasodilating activity, is used for the treatment of hypertension, ischemic heart disease and congestive heart failure. Carvedilol is administered as a racemic mixture of the *R*(+)- and the *S*(−)-enantiomer. The enantiomers exhibit different pharmacological effects, the β-receptor blocking activity of the *S*-enantiomer is about 200-fold higher than that of *R*-carvedilol, whereas both enantiomers are equipotent α-blockers [1]. After ingestion, carvedilol is extensively metabolized in the liver [2], main metabolites are the *O*-demethylated carvedilol (DMC) and the two ring-hydroxylated metabolites 4- and 5-hydroxyphenyl carvedilol (4-OHC, 5-OHC). Metabolism of racemic carvedilol is stereoselective giving rise to different bioavailabilities of the enantiomers of about 15% for *S*-carvedilol and about 31% for *R*-carvedilol [1]. In order to get information about

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pharmacokinetic–pharmacodynamic relations, it is therefore necessary to measure individual carvedilol enantiomer concentrations.

HPLC methods using chiral derivatization have been developed to determine the enantiomers of carvedilol in human plasma [3,4]. The amounts of 0.5–1 ml plasma needed for the sample preparation is too large for pharmacokinetic studies in young children where the blood volume collected should be kept to a minimum. Our aim was to develop a sensitive method for the separation of carvedilol enantiomers requiring only small sample amounts. The capillary electrophoresis technique has obtained increasing importance in analytics, especially in the separation of chiral compounds [5]. Advantages compared to conventional HPLC methods are the small sample amounts needed, the sample preparation not requiring chiral derivatization and the low demand of solvents.

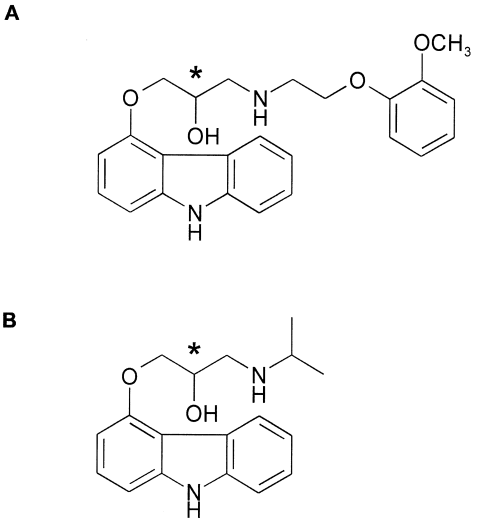

Fig. 1. Chemical structures of carvedilol (A) and the internal standard carazolol (B). The asterisks indicate the asymmetrical carbon atoms.

2. Experimental

2.1. Chemicals and material

All reagents used were of analytical grade unless otherwise indicated. Racemic carvedilol (Hoffmann–La Roche, Grenzach–Wyhlen, Germany; Fig. 1A) was used as certified reference compound for quantitative analysis. Carazolol (Klinge Pharma, München, Germany; Fig. 1B) was used as internal standard. The metabolites of carvedilol were a gift from Professor E. Mutschler (Institute of Pharmacology, University of Frankfurt, Germany). Succinyl-β-cyclodextrin (D.S. 0.4) and methyl-α-cyclodextrin (D.S. 1.8) were purchased from Wacker Chemie (Burghausen, Germany), hydrochloric acid (HCl) and boric acid from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH), glacial acetic acid and phosphoric acid 85% were obtained from Baker (Deventer, The Netherlands), diethylether from Baker Chemical (Phillipsburg, NJ, USA). Water was purified by bidestillation in the laboratory. Nitrogen was purchased from Messer Griessheim (Griessheim, Germany). Blank plasma was obtained from fasted healthy volunteers. For sample rotation an Ika-Vibrax-VXR (Janke and Kunkel, Staufen im Breisgau, Germany) was used, the samples were centrifuged in a Labofuge III (Heraeus Christ, Osterode am Harz, Germany).

2.2. Preparation of reagents and stock solutions

Racemic carvedilol was dissolved in 0.025 *M* HCl to a concentration of 50 mg/l. Various working solutions with concentrations between 5 and 0.16 mg/l were prepared. The internal standard carazolol was dissolved in 0.025 *M* HCl to a concentration of 10 mg/l. For plasma analysis an internal standard solution of 100 μg/l was used. Electrophoresis buffer was prepared with 7.5 mg/ml succinyl-β-cyclodextrin and 15 mg/ml methyl-α-cyclodextrin in 150 m*M* phosphate buffer at a pH of 3.0, the cathode buffer consisted of 150 m*M* phosphate buffer at a pH of 3.0 without cyclodextrins. All stock solutions and working solutions were stored at 4°C. The Britton–Robinson buffer consisted of 6.18 g/l boric acid, 5.70 ml/l glacial acetic acid and 6.82 ml/l phosphoric acid 85% adjusted to a pH of 8.0 with 0.5 *M* NaOH.

2.3. Sample preparation

Blood was centrifuged at 4°C with 2740 *g* and the plasma samples were stored at −80°C until analysis.

To 100 μl of plasma, 100 μl of Britton–Robinson buffer (pH 8.0) and 20 μl of internal standard solution were added. The solutions were vortexed for 10 s before extraction with 1.1 ml of diethylether. The extraction procedure consisted of 5-min rotation followed by 5-min centrifugation at 2740 *g*. The supernatant was evaporated to dryness under a stream of nitrogen at ambient temperature. The residues were reconstituted in 20 μl of 0.025 *M* HCl, centrifuged at 2740 *g* for 5 min and an aliquot of about 36 nl was injected hydrodynamically into the capillary.

2.4. Instrumentation

Capillary electrophoresis (CE) analysis was performed on a Beckman P/ACE 2100 instrument with a laser-induced fluorescence (LIF) detector (Beckman Instruments, Munich, Germany). The CE-LIF instrument was run in an air-conditioned room (20–25°C). Fluorescence excitation was provided by an Omnichrome helium–cadmium laser (Series 74, Laser 2000, Wessling, Germany) with 20-mW and 325-nm excitation wavelength. Laser and detector were connected with an optical fiber (Omnichrome POS FDS A 1/2, Laser 2000, Wessling, Germany). The energy measurable at the end of the detector's fiber optics was 7 mW. Emission light was filtered through a 366-nm filter with a half bandwidth of 10 nm. Data were collected and integrated using the software Gold version 7.11 (Beckman System, Munich, Germany). A fused-silica capillary of dimension 37 cm×50 μm was employed. The liquid cooling system of the capillary was set to 20°C. The capillaries were initially preconditioned, prior to their first use, by rinsing them for 60 min with 0.1 *M* NaOH. Prior to storage, the capillary was flushed for 4 min with 0.1 *M* NaOH, for 4 min with double destilled water and finally for 2 min with air/nitrogen.

2.5. Assay

All rinsing stages were carried out with 20 p.s.i. (1 p.s.i.=6894.76 Pa) unless otherwise indicated. Before starting a series the laser was ignited at least 30 min in advance. Three pre-analyses were performed to allow the system to settle. Prior to each sample analysis, the system was programmed to rinse the capillary for 2 min with 0.1 *M* sodium hydroxide solution, for 2 min with methanol, for 0.5 min with double destilled water and for 0.5 min with electrophoresis buffer. Injection was performed hydrodynamically for 25 s with 0.5 p.s.i. resulting in an injection volume of about 36 nl. During injection the opposite end of the capillary was already located in the electrophoresis buffer vial. Separation was carried out by applying a voltage of 16 kV with normal polarity; this corresponds to 432 V/cm. The current was then approximately 85 μA. The migration times were 8.7 min for the internal standard, 11.2 and 11.5 min for *R*- and *S*-carvedilol, respectively. With a separation time of 12.0 min, the rinse stages and injection, the total time of analysis per sample was 17.5 min. Taking into account different detector responses to the enantiomers, the evaluation was based on peak areas corrected relative to the migration times [6].

2.6. Calibration

Solutions of different racemic carvedilol concentrations analysed with the CE-LIF method reproducibly gave ratios of corrected peak areas of 1:1 for the enantiomers. No conversion of the carvedilol enantiomers through the sample treatment process was observed. Calibration samples therefore were obtained by spiking 1 ml of blank plasma with 20 μl of the working solutions (Section 2.2.) of racemic carvedilol to yield six concentrations of each enantiomer in the range of 1.56–50 μg/l. The samples were stored at −20°C. They were processed further as described in Section 2.3. and analyzed in one sequence. Calibration curves according to the internal standard method were obtained by plotting concentrations versus relative to the migration times corrected peak area ratios.

2.7. Validation

To evaluate accuracy and precision, six spiked samples at 3, 6, 25, 100 μg/l of racemic carvedilol, resulting in carvedilol enantiomer concentrations of 1.56, 3.13, 12.5 and 50 μg/l, were analysed within one day to assess the intra-day variability. The procedure was repeated on three days to investigate

the inter-day precision and accuracy. The accuracy should be less than ±10% and the precision less than 10% in the working range. The limit of quantification (LOQ) was determined by analysing six samples independent of standards and by determination of precision and accuracy. Precision near the LOQ was allowed to be ≤20%, accuracy less than ±20% [7]. The limit of detection (LOD) was evaluated in a different experiment based on a signal-to-noise ratio of 3:1 (data not shown). A total absence of matrix interference was confirmed by analysis of blank plasma. In addition, the metabolites were injected. Their peaks did not interfere with those of *R*- or *S*-carvedilol (data not shown).

2.8. Application

The carvedilol enantiomer concentrations were determined in plasma samples taken from a 5-year-old patient treated with carvedilol for congestive heart failure. Thirteen blood samples were drawn before and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 8 h after oral administration of a test dose of 0.09 mg/kg body weight. This procedure had been approved by the local Ethics Committee (Ethik-Kommission der Ärztekammer Hamburg, No. 1292), the patient and his parents gave informed consent.

3. Results and discussion

3.1. Capillary electrophoresis

A capillary electrophoretic method with laser-induced fluorescence detection (CE-LIF) has been established for the determination of carvedilol in 100 μl plasma. Upconcentration of 1:5 was obtained by reconstitution of the extraction residue with only 20 μl of 0.025 *M* HCl. Thus, a lower LOQ could be reached. In order to guarantee reproducibility, an internal standard (carazolol) was used because of possible experimental differences in sample preparation, sample injection, separation and detection. Using a 30/37-cm fused-silica capillary and cyclodextrins for chiral selection, *R*-, *S*-carvedilol and the internal standard were readily separated within 12 min in plasma samples (Fig. 2A,B). Absence of any interference in all electropherograms was proven by injecting blank plasma (Fig. 2C) as well as the metabolites, which showed divergent migration times compared to those of the internal standard carazolol and the carvedilol enantiomers (data not shown).

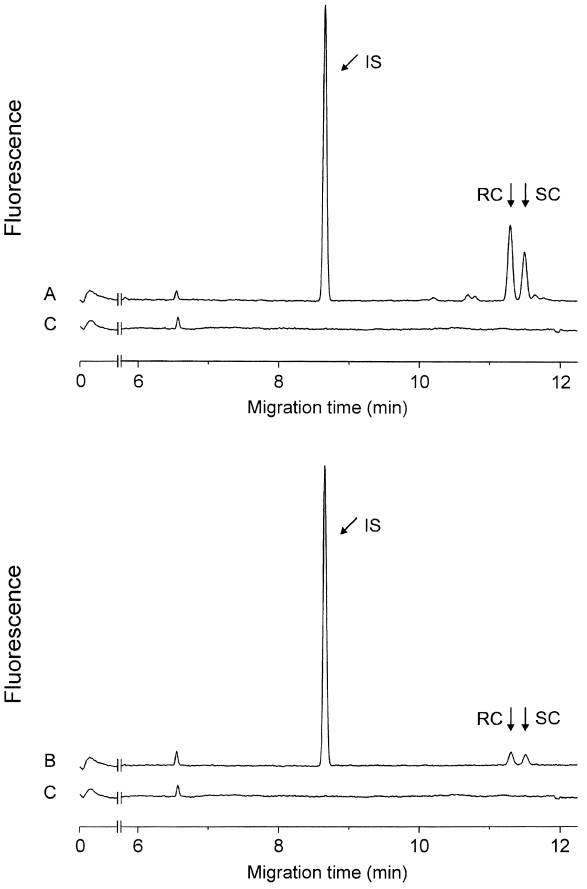

Fig. 2. Representative electropherograms of blank plasma (C) and plasma samples of a 5-year-old patient at 0.75 h (A: *R*-carvedilol, 10.5 μg/l; *S*-carvedilol, 6.54 μg/l) and 6 h (B: *R*-carvedilol, 1.58 μg/l; *S*-carvedilol, 1.39 μg/l) after oral administration of 0.09 mg/kg body weight of racemic carvedilol. Arrows indicate the peaks of the internal standard (I.S.), of *R*-carvedilol (RC) and *S*-carvedilol (SC).

3.2. Calibration and validation

The calibration curves were obtained by plotting the concentrations versus the corrected peak area ratios. The relations between the corrected peak area ratios and the *R*- and *S*-carvedilol concentrations

were $y=0.028x-0.004$ and $y=0.028x-0.006$, respectively, with correlation coefficients of 0.999, each ($n=6$, each, at four concentrations in the range of 1.56–50 μg/l *R*- and *S*-carvedilol, respectively). The method was linear over the whole concentration range of 1.56–50 μg/l carvedilol enantiomer in plasma using the internal standard method.

The results of the validation experiments are presented in Table 1 for *R*-carvedilol and in Table 2 for *S*-carvedilol. The LOD was 1 μg/l per carvedilol enantiomer based on a signal-to-noise ratio of 3:1 (data not shown). The LOQ for 100-μl plasma samples was determined to 1.56 μg/l for each carvedilol enantiomer. At the LOQ (1.56 μg/l), carvedilol enantiomers showed accuracy values between 96.6–112% (*R*-carvedilol) and 101–113% (*S*-carvedilol). Precision values expressed as percent coefficient of variation varied between 8.22–11.3% and 3.30–9.31%, respectively. In the working range at concentrations of 3.13, 12.5 and 50 μg/l of each carvedilol enantiomer, accuracy was between 90.5 and 107% for *R*-carvedilol and between 92.1 and 106% for *S*-carvedilol. Precision throughout the whole working range was 1.41 to 9.46% for *R*-carvedilol and 2.29 to 9.54% for *S*-carvedilol.

Table 1
Intra- and inter-day accuracy and precision of *R*(+)-carvedilol in human plasma

	Nominal concentrations of *R*(+)-carvedilol in plasma (μg/l), calibration range 1.56–50.0			
	1.56	3.13	12.5	50.0
Concentration found (arithm. mean value) (μg/l)				
Day 1 ($n=6$)	1.75	2.98	12.3	51.7
Day 2 ($n=6$)	1.51	2.90	11.3	53.3
Day 3 ($n=6$)	1.61	3.26	12.3	50.0
Inter-day ($n=18$)	1.62	3.05	12.0	51.7
Accuracy (arithm. mean value) (%)				
Day 1 ($n=6$)	112	95.5	98.6	103
Day 2 ($n=6$)	96.6	92.7	90.5	107
Day 3 ($n=6$)	103	104	98.2	100
Inter-day ($n=18$)	104	97.6	95.8	103
Precision (arithm. mean value) (C.V., %)				
Day 1 ($n=6$)	8.38	6.63	9.46	4.03
Day 2 ($n=6$)	8.22	8.36	5.86	1.41
Day 3 ($n=6$)	11.3	6.86	7.36	2.66
Inter-day ($n=18$)	10.9	8.64	8.34	3.81

3.3. Application

The CE-LIF method was used to measure carvedilol enantiomers in plasma of a 5-year-old patient receiving carvedilol for congestive heart failure due to dilated cardiomyopathy. With 100–200 μl of plasma, *R*- and *S*-carvedilol were determined at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 8 h after oral administration of a test dose of 0.09 mg/kg of racemic carvedilol. Maximum plasma concentrations of *R*- and *S*-carvedilol were 10.5 and 6.54 μg/l at 0.75 h after administration and minimum concentrations 8 h after administration were 0.80 and 0.78 μg/l, respectively. The plasma concentration time profiles of *R*- and *S*-carvedilol of this patient are illustrated in Fig. 3. Fig. 2A,B show representative electropherograms of this patient's plasma samples at 0.75 (A) and 6 h (B) after oral administration of 0.09 mg/kg of racemic carvedilol.

Heart failure due to dilated cardiomyopathy is a rare disease in pediatric patients but those patients have a poor prognosis and heart transplantation might be the last option [8]. Therefore, β-receptor blockers like carvedilol should improve morbidity and mortality of pediatric patients with heart failure comparable to their use in adults avoiding the need for heart transplantation [9]. In order to ensure safe

Table 2
Intra- and inter-day accuracy and precision of *S*(−)-carvedilol in human plasma

	Nominal concentrations of *S*(−)-carvedilol in plasma (μg/l), calibration range 1.56–50.0			
	1.56	3.13	12.5	50.0
Concentration found (arithm. mean value) (μg/l)				
Day 1 ($n=6$)	1.76	3.03	12.8	51.9
Day 2 ($n=6$)	1.57	3.07	11.5	52.9
Day 3 ($n=6$)	1.70	3.30	12.1	50.1
Inter-day ($n=18$)	1.68	3.13	12.1	51.6
Accuracy (arithm. mean value) (%)				
Day 1 ($n=6$)	113	96.9	102	104
Day 2 ($n=6$)	101	98.3	92.1	106
Day 3 ($n=6$)	109	106	96.8	100
Inter-day ($n=18$)	107	100	97.1	103
Precision (arithm. mean value) (C.V., %)				
Day 1 ($n=6$)	5.58	4.71	4.36	5.81
Day 2 ($n=6$)	3.30	2.29	6.38	6.33
Day 3 ($n=6$)	9.31	7.13	9.54	2.82
Inter-day ($n=18$)	7.91	6.32	7.86	5.47

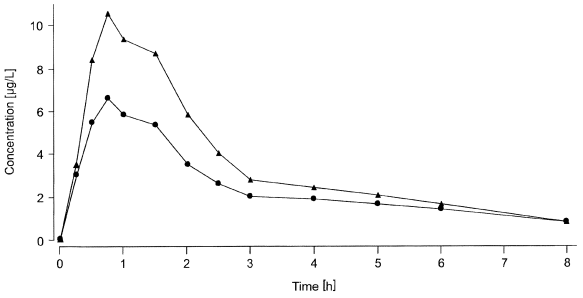

Fig. 3. Plasma concentration time profiles of *R*-carvedilol (▲) and *S*-carvedilol (●) in a 5-year-old patient after oral administration of 0.09 mg/kg body weight of racemic carvedilol.

and effective drug treatment in pediatric patients, pharmacokinetic investigations are mandatory.

3.4. Method development

Koppenhoefer et al. [10] showed a separation of carvedilol enantiomers in a general investigation about chiral separation of different drugs in solution. They used a 100 m*M* phosphate buffer at pH 2.5 with addition of 15 m*M* (corresponding to 17 mg/ml) β-cyclodextrin (β-CD) as anode buffer. We optimized this buffer for our system to a 150 m*M* phosphate buffer containing 7.5 mg/ml succinyl-substituted-β-CD. Analysing biological fluids, interference of all metabolites had to be taken into account since concentrations of the metabolites of up to one-tenth of the mother compound concentration are likely [1]. With this buffer, the peaks of the metabolites 4-OHC and 5-OHC, migrating earlier, did not interfere with the carvedilol enantiomer peaks. Carvedilol and the metabolite DMC were separated into enantiomers, but the two enantiomer pairs were not separated from each other, the *S*-enantiomer of carvedilol comigrated with one DMC enantiomer. In order to separate the DMC enantiomers from those of carvedilol, native α-CD was tested but did not separate carvedilol into enantiomers. Use of γ-CD only resulted in partial separation of carvedilol. Therefore, combination of the previously used succinyl-β-CD with methyl-substituted α-CD was tested. Preliminary studies showed that methyl-α-CD does not influence the separation of carvedilol and DMC into enantiomers, but alters the migration times. This was used to shift the two enantiomer pairs against one another in order to separate the DMC– from the carvedilol–enantiomer pair. At a concentration of 15 mg/ml of methyl-α-cyclodextrin added to the succinyl-β-CD containing buffer, *S*-carvedilol was baseline separated from the DMC enantiomers. By changing the buffer pH to 3.0, the separation time could be shortened without loss of separation strictness. The chiral internal standard carazolol was not separated into enantiomers under these conditions.

Instead of UV-detection, used by Koppenhoefer et al. [10], we employed a laser-induced fluorescence method providing a more sensitive and more selective detection. This is of particular advantage when analysing small volumes of biological samples like in pharmacokinetic studies of young children.

4. Conclusion

A sensitive capillary electrophoretic method to determine carvedilol enantiomers in very small volumes of human plasma with good precision and accuracy has been developed. As main metabolites, 4- and 5-hydroxyphenyl carvedilol are migrating faster and *O*-demethyl carvedilol is migrating slower than the carvedilol enantiomers, all of these metabolites are well separated from the peaks of carvedilol [11]. This method might be extended to the quantification of other racemic β-receptor blockers with related chemical structure.

As shown, this method allows to determine plasma concentration time profiles of carvedilol enantiomers in young patients with congestive heart failure who receive only a test dose of racemic carvedilol at the beginning of the β-blocker therapy. With this method, several questions can be addressed in future work. It is not known whether pharmacokinetics of the two carvedilol enantiomers develop differently from infancy to adulthood. Furthermore, pharmacokinetic–pharmacodynamic (PK/PD) relations can be investigated by correlating the enantiomer concentrations to the corresponding pharmacodynamic effects in infants and children. Thus, this method will especially be useful in pediatric patients where only small sample volumes are available.

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