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Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 407–412
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Comparison between capillary electrophoresis and high-performance liquid chromatography for the stereoselective analysis of carvedilol in serum

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Received 8 January 2001; received in revised form 3 August 2001; accepted 4 September 2001

Abstract

A high-performance liquid chromatographic (HPLC) assay using a chiral stationary phase was developed and validated for the determination of carvedilol enantiomers in human serum and was compared with a previously developed capillary electrophoresis (CE) method. The CE and the HPLC assay were compared by analyzing a series of serum samples containing racemic carvedilol in different concentrations using the two methods. The concentrations obtained by the two assays were not found to be significantly different indicating that CE and HPLC are comparable in terms of reproducibility and precision for the stereoselective analysis of carvedilol in human serum.

Keywords: High-performance liquid chromatography; Stereoselective analysis; Carvedilol; Capillary electrophoresis

1. Introduction

The resolution power of capillary electrophoresis (CE) has now been widely recognized as a valuable alternative to conventional methods for the analysis of drug enantiomers. Compared with high-performance liquid chromatography (HPLC), it offers the advantages of faster method development, higher efficiencies and lower consumption of solvents and reagents. In a previous report [1], we described the development of a CE assay for the analysis of carvedilol enantiomers in serum using hydroxypropyl-β-cyclodextrin as the chiral selector.

Carvedilol (Fig. 1) is a new antihypertensive agent with non-selective β- and α_1-adrenergic receptor blocking activities [2–4] which is also being used in the treatment of congestive heart failure [5–7]. After a 12.5 mg i.v. short infusion, Spahn et al. [8] reported a C_{max} of 70 and 89 ng/ml, for (*S*)-(−)- and (*R*)-(+)-carvedilol, respectively. The C_{max} values obtained by the same group, after oral administration of 50 mg of drug, were 21 and

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0731-7085/03/$ - see front matter
PII: S0731-7085(02)00609-X

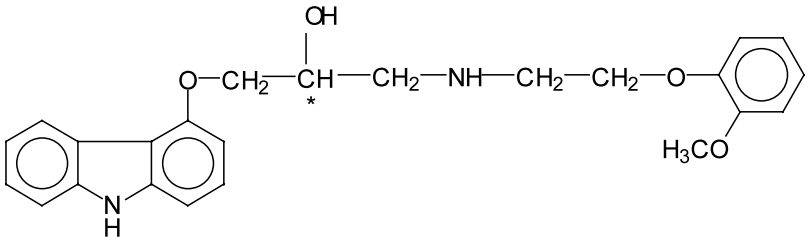

Fig. 1. Chemical structure of carvedilol. The asterisk denotes the chiral center.

78 ng/ml, for (*S*)-(−)- and (*R*)-(+)-carvedilol, respectively [8]. Similar values were also obtained by Neugebauer et al. [9]. The C_{max} values reported by Fujimaki et al. [10] after an oral dose of 20 mg of carvedilol were much lower, only 10 and 25 ng/ml, for (*S*)-(−)- and (*R*)-(+)-carvedilol, respectively. Therefore, a sensitive method is needed to study the stereoselective pharmacokinetics of carvedilol.

The current analytical methods for carvedilol enantiomers involve derivatization with optically pure reagents to form diastereomers and separation by HPLC using either adsorption or partition modes [8–13]. We describe in this report a new HPLC method for carvedilol enantiomers, which does not require derivatization of the drug and uses a chiral stationary phase to produce the resolution of the enantiomers.

HPLC is an established analytical method widely used for chiral separations. Therefore, the objective of this study is to compare CE with HPLC as a means of validating the CE assay. As pointed out by Altria et al. [14], agreement between two different separation techniques reinforces the validity of the method under investigation.

2. Experimental

2.1. Materials and supplies

R,*S*-(±)-Carvedilol, (*R*)-(+)-carvedilol, and (*S*)-(−)-carvedilol were kindly donated by Boehringer Mannheim GmbH (Mannheim, Germany). (−)-Propranolol was obtained from Ayerst Laboratories (Montreal, Que.). All other chemicals and reagents were of analytical grade. Fused silica capillaries, uncoated, 57 cm in length (50 cm effective length), 75 μm ID and hydroxypropyl-β-cyclodextrin were purchased from Beckman (Palo Alto, CA). Samples were filtered through 0.2 μm Acrodisc LC13 PVDF filters (Gelman, Ann Arbor, MI) prior to injection into the CE apparatus. Ethyl alcohol, reagent, denatured (5%, v/v, isopropyl alcohol), HPLC grade, was from Sigma (St. Louis, MO). Hexane and dichloromethane, HPLC grade, and ethyl ether were obtained from Fisher Scientific (Nepean, Ont.). The chiral HPLC column, (*S*)-indoline-2-carboxylic acid and (*R*)-1-(α-naphthyl)ethylamine (3022), 250 × 3.2 mm ID, 5 μm particle size was purchased from Phenomenex (Torrance, CA).

2.2. Equipment

CE was performed on a P/ACE System 5000 instrument (Beckman Instruments, Fullerton, CA). Data were collected and analyzed on a 486/66 Hz computer (Dell Computer Corporation, Austin, TX) equipped with System Gold Chromatography Software, version 8.10 (Beckman Instruments, Fullerton, CA).

Chromatographic analyses were performed using a Tosohaas TSK-6010 HPLC precision pump (Japan), a Shimadzu SIL-9A autoinjector, a Shimadzu RF-551 fluorescence detector and a CR501 Chromatopac Shimadzu integrator (Kyoto, Japan).

2.3. Electrophoretic conditions

Electrophoresis was performed using a fused silica capillary, 75 μm ID, 57 cm (50 cm to the

detector). The background electrolyte consisted of 25 mM sodium phosphate buffer, pH 2.5, and 10 mM hydroxypropyl-β-cyclodextrins. The injection was by pressure at 0.5 psi for 4 s (approximately 18 nl). Separation voltage was 18 kV, with the cathode being at the detector end. The capillary was rinsed between runs with 0.1 M sodium hydroxide (1 min), water (0.5 min), 0.1 M hydrochloric acid (0.5 min) and water (0.5 min). Before injection, the capillary was rinsed with the run buffer for 1 min. Detection was by UV at 200 nm. The internal standard was (−)-propranolol. Corrected peak area ratios (enantiomer/internal standard) were used for quantitations.

2.4. Chromatographic conditions

The chiral chromatographic assay used (*S*)-indoline-2-carboxylic acid and (*R*)-1-(α-naphthyl)ethylamine (Phenomenex 3022, Torrance, CA) as the stationary phase (250 × 3.2 mm ID, 5 μm). The mobile phase consisted of hexane:dichloromethane:HPLC grade ethanol (50:35:15, v/v/v), and 0.25% (v/v) trifluoroacetic acid at a flow rate of 0.55 ml/min. The injection volume was 10 μl. The internal standard used was (−)-propranolol and peak area ratios were used for quantitations. The detection was by fluorescence at 284 nm for excitation and 343 nm for emission.

2.5. HPLC analysis of carvedilol enantiomers extracted from human serum using a phenomenex 3022 chiral stationary phase

2.5.1. Determination of detector linearity for carvedilol enantiomers in serum

Calibration samples of carvedilol in human serum were prepared in duplicate using stock solutions of racemic carvedilol in deionized water. Aliquots of the solutions were added to serum to obtain, at the low-concentration range (for plasma levels determinations), final concentrations of 1, 2.6, 5.2, 7.3, 15.6, 31.2, and 62.4 ng/ml of each enantiomer. Fifty microliters of internal standard solution (0.4 μg/ml (−)-propranolol in HPLC grade ethanol) was added to the samples (0.5 ml) which was then submitted to protein precipitation with 10% trichloroacetic acid, basification with 1 M sodium hydroxide and extraction with ethyl ether. The extracts were evaporated to dryness under nitrogen and reconstituted in 0.1 ml HPLC grade ethanol for injection.

At the high-concentration range (for protein binding studies), racemic carvedilol was added to human serum to obtain final concentrations of 1, 2, 3, 4, and 5 μg/ml of each enantiomer. An aliquot (100 μl) of internal standard solution (4 μg/ml (−)-propranolol in HPLC grade ethanol) was added to the samples (0.2 ml serum), which were then extracted as described above.

2.5.2. HPLC intra- and inter-assay variability

The reproducibility of the HPLC assay for the determination of carvedilol enantiomers in serum was determined by analyzing triplicate serum samples to which racemic carvedilol was added to obtain final concentrations of 2 and 4 μg/ml per enantiomer. Samples were extracted and analyzed as described above. Peak area ratios of carvedilol enantiomer/internal standard were calculated. Intra-assay variability was determined in terms of coefficient of variation (CV) in percent of the three determinations. Inter-assay variability was obtained by performing the same determinations on three different days.

2.5.3. Comparison between the HPLC and the CE assays

Repeated calibration curves of carvedilol enantiomers in human serum in concentrations of 1, 2, 3, 4, and 5 μg/ml per enantiomer were prepared. Samples for HPLC analysis (0.2 ml) were extracted and analyzed using the HPLC method described above. Samples for CE analysis (0.7 ml) were extracted and analyzed as described in a previous report [1]. The slopes and intercepts obtained for each calibration curve were used to calculate the concentration of carvedilol enantiomers in each sample. A graph of the concentrations obtained by CE versus those obtained by the HPLC method was constructed and the correlation coefficient was determined.

3. Results and discussion

3.1. Determination of detector linearity for carvedilol enantiomers in human serum

Linearity of detector response in the serum concentration range 1–62 ng/ml per enantiomer, which reflects the serum levels achieved after carvedilol administration, showed excellent detector linearity, with coefficients of determination (r^2) of 0.996 and 0.999 for (*R*)-(+)- and (*S*)-(−)-carvedilol, respectively.

The limit of quantitation (LOQ) of the direct chiral HPLC was 1 ng/ml for each enantiomer in serum (signal-to-noise ratio of 10). Thus, the native fluorescence of carvedilol provided a highly sensitive assay for the determination of the drug in serum. The LOQ obtained is about the same as the values reported in the literature, with assays involving diastereomer formation [8–13].

Linearity of detector response was also obtained at the high serum concentration range 1–5 μg/ml per enantiomer (to be used in in vitro protein binding studies), with r^2 higher than 0.999 for both carvedilol enantiomers.

3.2. HPLC intra- and inter-assay variability

The reproducibility of the HPLC assay for the determination of carvedilol enantiomers in serum using the chiral column Phenomenex 3022 was determined. The results are presented in Table 1. Intra- and inter-assay variabilities under 6%, expressed as CV, were obtained.

3.3. Comparison between the HPLC and the CE assays

A representative electropherogram and a chromatogram from an extraction of racemic carvedilol (2 μg/ml) from human serum are shown in Figs. 2 and 3, respectively. Good resolution (Rs = 1.5 by CE and 1.2 by HPLC) of the enantiomers was obtained by both methods with no interference from the matrix.

Serum concentration values obtained by the CE assay correlated well with those obtained by HPLC, with a correlation coefficient (r) of 0.995. A graph of the correlation between the concentrations obtained by the two methods is presented in Fig. 4. The parameters of the line obtained (value ± confidence limit) were slope $b = 0.990 \pm 0.044$ and intercept $a = 0.057 \pm 0.151$. Statistical analysis (Student's t-test) of the parameters obtained for the correlation line indicates that the slope is not different from the theoretical slope of unity ($t = 0.477$, $P > 0.50$), and the intercept is not different from zero ($t = 0.785$, $P > 0.20$; $n = 25$). Good correlation between CE and HPLC was also reported by Pruñonosa et al. [15] for the determination of cicletanine in plasma.

In terms of analysis times, CE offers substantial advantages compared with HPLC. In the present method, the analysis time required for enantiomer resolution using the CE method was about 15 min plus a rapid conditioning cycle with five consecutive rinses (total rinse time = 3.5 min) to ensure reproducibility of the assay and also to remove serum components which tend to adhere to the capillary wall. Thus, the total analysis time was 18.5 min for CE, whereas for the HPLC assay, an analysis time of 27 min was necessary. While the

Table 1
Intra- and inter-assay CV (%) for the determination of carvedilol enantiomers in human serum by HPLC

Concentration (μg/ml)	Intra-assay CV[a] (%)		Inter-assay CV[b] (%)	
	(*R*)-(+)	(*S*)-(−)	(*R*)-(+)	(*S*)-(−)
2	2.1	5.7	4.2	5.1
4	3.6	5.6	5.6	5.7

[a] $n = 4$ determinations.
[b] $n = 3$ days.

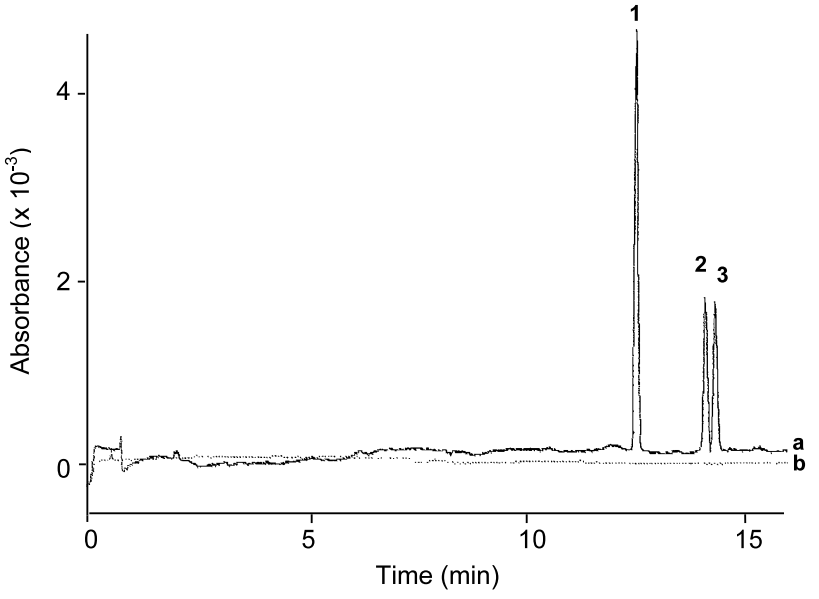

Fig. 2. Electropherogram of an extraction from human serum. (a) Serum containing (±)-carvedilol (2 μg/ml) and internal standard. (b) Blank serum: (1) (−)-propranolol, (2) (*R*)-(+)-carvedilol, and (3) (*S*)-(−)-carvedilol. Electrophoretic conditions: background electrolyte—25 mM sodium phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; capillary—fused silica, uncoated, 57 (50) cm length, 75 μm ID; voltage—18 kV; temperature—20 °C; injection—pressure at 0.5 psi for 4 s; detection—UV at 200 nm.

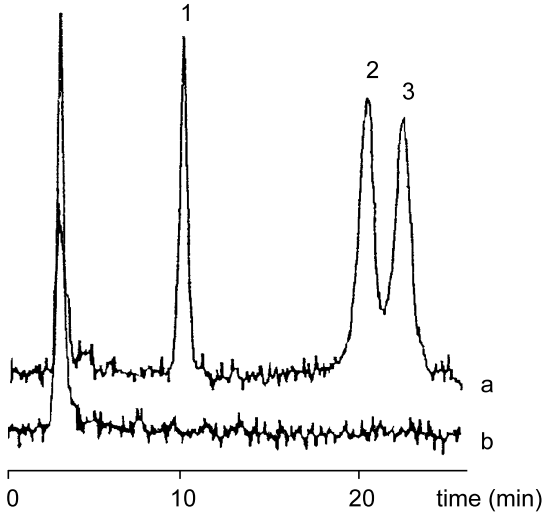

Fig. 3. Chromatogram of an extraction from human serum. (a) Serum containing (±)-carvedilol (2 μg/ml) and internal standard. (b) Blank serum: (1) (−)-propranolol, (2) (*R*)-(+)-carvedilol, and (3) (*S*)-(−)-carvedilol. Chromatographic conditions: HPLC column—(*S*)-indoline-2-carboxylic acid and (*R*)-1-(α-naphthyl)ethylamine, 250 mm length × 3.2 mm ID, 5 μm; mobile phase—hexane:dichloromethane:ethanol:trifluoroacetic acid (50:35:15:0.25, v/v/v/v); flow rate—0.55 ml/min; detection—fluorescence; 284 nm (EX)/343 nm (EM).

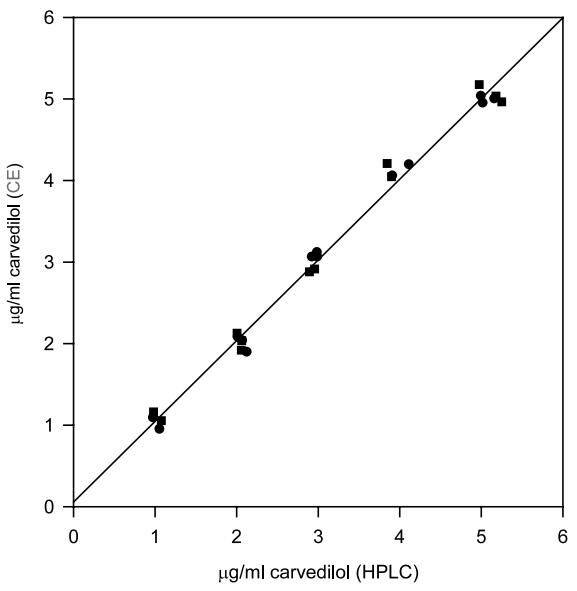

Fig. 4. Correlation graph of serum concentration values for (*R*)-(+)-carvedilol (●) and (*S*)-(−)-carvedilol (■), obtained by CE and HPLC. Line parameters: correlation coefficient $r = 0.995$; slope $b = 0.990$ (not different from the theoretical slope of unity, $t = 0.477$, $P > 0.50$); intercept $a = 0.057$ (not different from zero, $t = 0.785$, $P > 0.20$; $n = 25$).

HPLC chiral column required an equilibration time of about 1 h prior to analysis, this is not required with the CE technique.

Overall, method development was much faster with CE since it did not require extensive equilibration times compared with the chiral HPLC columns. Several buffer systems can be tested in CE in less than 1 h. The low consumption of solvents and buffer additives is also a major advantage in CE. Only 10 ml of buffer was sufficient to perform about 20 determinations. The chiral selectors used in CE are also relatively less expensive than the chiral HPLC columns.

One of the principal limitations of CE is detectability. Due to the extremely small diameter of the capillary, volumes of the order of nanoliters are typically injected. For the same reason, the light path at the on-capillary detector window is very short, reducing the signal produced by the sample. For the chiral CE method developed for carvedilol in serum, an LOQ of 50 ng/ml per enantiomer was obtained [1] whereas HPLC provided an LOQ of 1 ng/ml. In terms of injection volumes, in HPLC, injections of 10 μl were used, whereas the injection volumes calculated for CE (pressure of 0.5 psi for 4 s) were of the order of 18 nl. The actual amounts of drug at the detector were approximately 0.003 and 0.04 ng of each enantiomer for CE and HPLC, respectively. Thus, it can be seen that the sensitivity at the detector was actually higher for CE compared with HPLC. It should also be noted that the detection systems used were different. While HPLC used fluorescence detection, detection in the CE method was by UV absorption. Hence, the sensitivity limitations of CE are related to the small quantities injected and consequently dependent on the concentration of the starting material. This is particularly significant in pharmacokinetic studies where drug concentrations are often extremely low, an example being carvedilol.

4. Conclusion

A simple and sensitive HPLC assay was developed for the determination of carvedilol enantiomers in serum. The method was compared with a previously developed CE method [1]. Results indicate that the CE method compares well with HPLC and can be used for the determination of carvedilol enantiomers in human serum. Although limits of quantitation are lower with HPLC, the CE assay offers the advantage of faster analysis times and low consumption of solvents.

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