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Measurement of carvedilol in plasma by high-performance liquid chromatography with electrochemical detection

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Abstract

Carvedilol is a β/α_1-adrenoceptor blocker. A sensitive method for measuring plasma levels of carvedilol in human administrated low doses is needed since its plasma concentration is low. We measured carvedilol and carvedilol M21-aglycon using high-performance liquid chromatography (HPLC) with electrochmical detection. The amperometric detector was operated at 930 mV versus Ag/AgCl. Mean coefficients of variation ($n = 5$) for carvedilol and M21-aglycon were 4.0 and 7.7% (intra) and 6.1 and 6.7% (inter), respectively. The lower limit of quantification for each analyte was 0.10 ng/ml (signal-to-noise ratio = 3). This lower limit of quantification for carvedilol was sufficient for clinical use.

Keywords: Electrochemical detection; Carvedilol

1. Introduction

Carvedilol (BM 14.190), (±)-1-(carbazol-4-yloxy)-3-[[2-(*O*-methoxyphenoxy)ethyl]amino]-2-propanol, which has been prescribed as an antihypertensive agent and as an antiangina agent [1,2], is accepted as an effective agent for the treatment of congestive heart failure (CHF) [3–5]. It causes noncardioselective β/α_1-adrenoceptor blockade and also has an anti-oxidant effect [6–8]. Carvedilol M21-aglicon, (±)-4-[2-hydroxy-3-[[2-(*O*-methoxyphenoxy)ethyl]amino]-propoxy]-9H-carbazol-3-ol, one of metabolites of carvedilol, has been reported to have a more potent anti-oxidant effect than the effects of other hydroxylated analogs of carvedilol and the parent compound [6,9]. An anti-oxidant effect is thought to be beneficial for patients with CHF since the effect protects the heart [10–12]. This analog works as an anti-oxidant agent at subnanomolar levels [14,17], though it has not been detected in human plasma.

The starting dose of carvedilol for the treatment of CHF is smaller than that used for treatment of hypertension. The dose is then gradually increased to the optimal dose [13]. The plasma concentration of carvedilol in heart failure patients is expected to be low at the start of treatment.

The aim of this study was to develop a sensitive method for measuring the level of carvedillol as well as the level of M21-aglycon, which has not been detected in human plasma. High-performance liquid chromatography with electrochemical detection (HPLC–ECD) has not been used for detection of these agents despite its sensitivity and redox behavior.

2. Experimental

2.1. Apparatus and column

The HPLC system consisted of an Eicom model PC-300 (Eicom, Kyoto, Japan) and a Rheodyne model 7725 injection (Rheodyne, Cotati, CA, USA) with a loop of 100 μl. The electrochemical detector was an Eicom model ECD-300 (Eicom, Kyoto, Japan) with a glassy carbon cell WE-GC (Eicom, Kyoto, Japan). It was operated in the dc mode at +930 mV versus an Ag/AgCl electrode. Chromatograms were recorded with a Hitachi model D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan). The column used was a 254 mm × 4.6 mm Chemcosorb column (Chemco, Tokyo, Japan). The temperature of the column was kept at 34 °C

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using an Lc column conditioner model 370 column oven (Chemco, Tokyo, Japan).

2.2. Reagents and solutions

Carvedilol (BM 14.190) and carvedilol M21-aglycon (BM 91.0228) were gifts from Daiichi Pharmaceutical Co. (Tokyo, Japan). Desipramine hydrochloride (Sigma) and all reagents used for assays were of the highest grade available. Water was obtained by Milli-RO and Milli-Q Millipore systems.

Stock standard solutions of carvedilol and carvedilol M21-aglycon were stocked at concentrations of 1 mM. These stock standard solutions were stored at −80 °C. The assay standards were prepared freshly in distilled water for each assay from the stock solutions.

2.3. Chromatographic conditions

The mobile phase consisted of 53% (v/v) methanol and 47% (v/v) phosphate buffer (pH 3.8), which are 65.2 mM citric acid and 69.6 mM disodium hydrogen phosphate. The buffer also contained 0.25 mM sodium *n*-octyl sulfate (SOS) (Nakalai Tesque, Kyoto, Japan) and 0.01 mM ethylenediaminetetra-acetic acid disodium salt (EDTA·2Na) (Wako, Tokyo, Japan). The mobile phase was pumped at a flow rate of 0.47 ml/min.

2.4. Extraction

About 5 ml of whole blood was collected into tubes containing 0.01 mM of EDTA. 2Na and spun at 1500 × *g* in a refrigerated centrifuge at 4 °C for 10 min. The plasma was stored at −80 °C until use.

One ml of plasma was mixed with 5 ng of desipramine dihydrochloride as an internal standard. The plasma to which had been added 100 μl of 0.1N NaOH, 900 μl of saturated NaCl and 0.6 mg of ascorbic acid [14] was extracted with 5 ml of diethylether (Wako, Tokyo, Japan). Plasma was shaken for 10 min at room temperature and placed in a freezer at −40 °C until the plasma layer had frozen. The agents in the ether layer were extracted into 200 μl of phosphate buffer (pH 2.8), 85.8 mM citric acid/28.4 mM disodium hydrogen phosphate, with shaking for 10 min at room temperature. The aqueous layer was injected into the HPLC system.

2.5. Subjects and medication

Three patients with CHF were enrolled in this study after obtaining informed consent. The patients had symptoms of CHF despite treatment with digoxin, diuretics and an ACE inhibitor. Each patient was given 2.5 mg of carvedilol orally, and blood samples were taken at 0, 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h after administration. One healthy volunteer was given 30 mg of carvedilol orally after overnight fasting, and a sample was taken 2 h after administration.

3. Results

3.1. Assay conditions

Table 1 shows absolute recoveries of carvedilol, M21-aglycon and desiplamine, which show how the anti-oxidant reagents protected against oxidative loss of analytes in whole blood and plasma during the assay procedure. The recoveries of each sample were determined against absolute peak height of known amount of each solution of the standards. The effects of 10 mM sodium disulfite ($Na_2S_2O_5$) and 37 mM ascorbic acid (VC) [14] added to whole blood immediately after sampling and the effects of those added to plasma during the assay procedure were compared. The addition of $Na_2S_2O_5$ resulted in decreased recovery of M21-aglycon. The addition of VC to plasma samples resulted in 20% greater recovery of carvedilol and M21-aglycon. We used the condition with number sign (#) throughout this study: VC was added only to the plasma during assay.

3.2. Mobile phase conditions

In order to determine the optimum potentials for carvedilol and carvedilol M21-aglycon, a hydrodynamic voltammogram was generated (Fig. 1). The maximum signal of each agent reached a plateau at the potential of 930 mV. Because of background current and noise, 930 mV was used throughout this study.

The pH of the mobile phase affected the peak current of carvedilol and M21-aglycon (Fig. 2). The maximum

Table 1
Absolute recoveries of carvedilol, M21-aglycon and desiplamine (internal standard) in samples

Anti-oxidant	Samples		Absolute recovery (%)		
	Whole blood	Plasma	M21-aglycon	Carvedilol	Desiplamine
Anti-oxidant	(−)	(−)	58	60	70
Sodium disulfite					
	(+)	(−)	44	65	73
	(+)	(+)	55	60	60
	(−)	(+)	60	67	78
Ascorbic acid					
	(+)	(−)	77	63	65
	(+)	(+)	84	64	70
	(−)#	(+)	92	75	72

Samples were prepared from drug free human plasma and whole blood spiked with 1 ng of each analyte. Whole blood (+) means 10 mM sodium disulfite ($Na_2S_2O_5$) or 37 mM ascorbic acid was added to the whole blood before the separation of plasma. Plasma (+) means 10 mM sodium disulfite ($Na_2S_2O_5$) or 37 mM ascorbic acid was added to the plasma before the extraction with organic layer during the assay procedure. We used the condition with number sign (#) throughout this study.

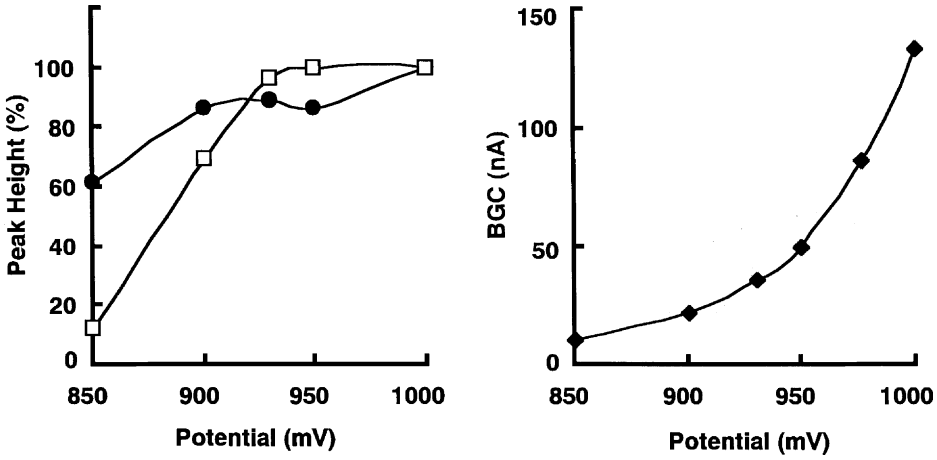

Fig. 1. A hydrodynamic voltammogram for electrochemical responses (peak heights) of 3.0 ng of carvedilol (□) and carvedilol M21-aglycon (●) at various potential settings. Peak height at 1000 mV was arbitrary determined to be 100%. BGC: background current.

peak current was detected near pH 3.8. The effects of pH on the k' values of these agents by ion-pairing reagents are shown in Fig. 3. As the pH decreased, one mM SOS increased the k' values of the agents when we examined them in the solution from pH 3.0 to 4.0 (Fig. 3b). A pH value of 3.8 was therefore selected for this study. The effects of various concentrations of SOS on the k' values of these agents under the mobile phase conditions (pH 3.8, 53% (v/v) methanol) are shown in Fig. 4. Retention was enhanced for carvedilol and M21-aglycon with increase in the concentration of SOS. The addition of 0.25 mM of SOS to the mobile phase caused satisfactory separation of peaks.

3.3. Calibration graphs and sensitivity

As shown in Table 2, the precisions of the values of analytes were reasonable for both intra-assays and intra-assays. Mean coefficients of variation ($n = 5$) for carvedilol and M21-aglycon were 4.0 and 7.7% (intra) and 6.1 and 6.7% (inter).

Calibration graphs in human plasma were linear in the range of 0.10–150.00 ng/ml for carvedilol and in the range of 0.10–3.40 ng/ml for M21-aglycon. The linear regression equations of each of analyte was obtained as follows: $y = (-0.03 \pm 0.04) + (0.26 \pm 0.01)x$ for intra-assay ($n = 5$, $r^2 = 1$) and $y = (-0.20 \pm 0.00) + (0.26 \pm 0.01)x$ for intra-assay ($n = 5$, $r^2 = 0.99$) for carvedilol; $y = (-0.01 \pm 0.00) + (0.15 \pm 0.01)x$ for intra-assay ($n = 5$, $r^2 = 1$) and $y = (-0.02 \pm 0.01) + (0.13 \pm 0.01)x$ for intra-assay ($n = 5$, $r^2 = 0.98$) for M21-aglycon; y is the peak-height ratio of each analyte to internal standard; x is the amount of each analyte in ng/ml and r^2 is the correlation coefficient.

The lower detection limit for each analyte was 0.10 ng/ml with a signal-to-noise ratio of 3. The data indicate good linearity of proposed method.

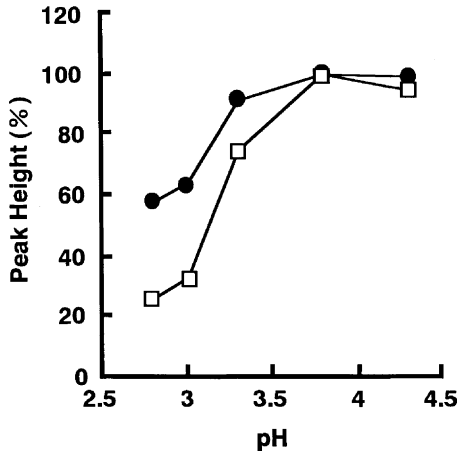

Fig. 2. Effects of mobile phase pH on peak heights of carvedilol and carvedilol M21-aglycon. Peak heights at pH 3.8 was arbitrary determined to be 100%. (□) carvedilol, (●) M21-aglycon.

Table 2
Precision and accuracy for the analysis of carvedilol M21-aglycon and carvedilol in human plasma

Actual value (ng/ml)	Observed value (ng/ml)		Coefficient variation (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
M21-aglycon				
0.1	0.11 ± 0.01	0.14 ± 0.01	9.4	5.1
1.0	0.98 ± 0.06	0.95 ± 0.09	5.8	9.3
3.4	3.40 ± 0.27	3.50 ± 0.23	8.0	5.7
Carvedilol				
0.1	0.15 ± 0.00	0.17 ± 0.01	1.3	4.0
1.0	4.80 ± 0.32	5.47 ± 0.49	6.8	9.0
150.0	150.00 ± 5.73	151.00 ± 7.81	3.8	5.2

Each amount of analyte spiked in drug free plasma sample. Intra-assay data were based on five replicate analyses and inter-assay data were from five consecutive days. Observed values were presented as mean ± standard deviation.

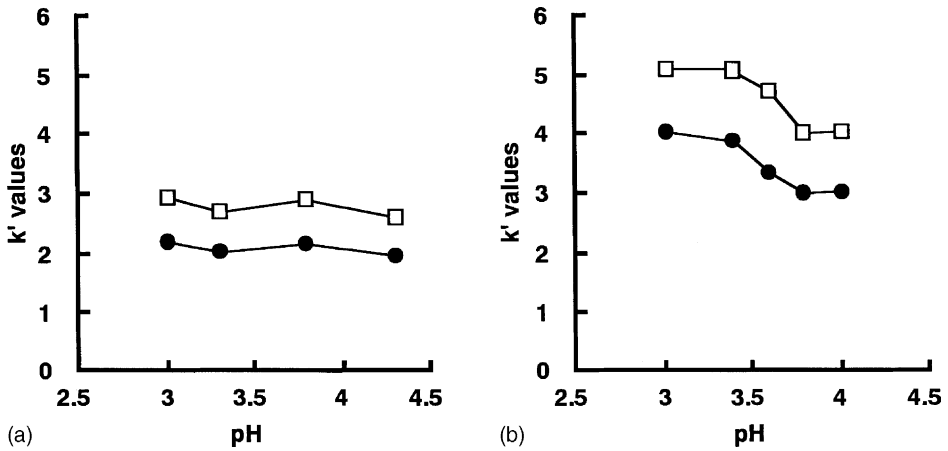

Fig. 3. Effects of mobile phase pH on the k' values of carvedilol and carvedilol M21-aglycon under mobile phase conditions of 53% (v/v) methanol: (a) without SOS and (b) with 1 mM of SOS. (□) carvedilol, (●) M21-aglycon.

3.4. Chromatograms

Under the conditions used for the mobile phase (pH 3.8, 53% methanol, 0.25 mM SOS), drug-free control human plasma showed no significant peaks in chromatograms at the retention times of carvedilol, M21-aglycon and the internal standard. The plasma from the healthy volunteer taken 2 h after oral administration of 30 mg of carvedilol showed satisfactory separation of peaks (Fig. 5b). Peaks 1–3 in this figure represent M21-aglycon, carvedilol and desiplamine as internal standard. The concentrations of carvedilol and M21-aglycon were 35.40 and 0.32 ng/ml.

3.5. Concentration–time curves of carvedilol

The concentrations of carvedilol in the plasma of three patients with CHF who had each been given 2.5 mg carvedilol were detected over a period of 24 h (Fig. 6). However, M21-aglycon was not detected. The plasma concentrations of carvedilol in the three patients at 24 h after administration of the agent were 0.93, 0.37 and 0.11 ng/ml.

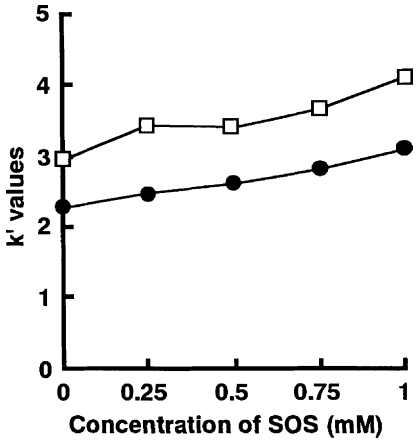

Fig. 4. Effects of sodium octyl sulfate (SOS) on the k' values of carvedilol and carvedilol M21-aglycon under mobile phase pH 3.8, 53% (v/v) methanol. (□) carvedilol, (●) M21-aglycon.

4. Discussion

There are various methods for measuring the plasma concentration of carvedilol using fluorometric detection [15–17]. Patients with CHF are usually administered carvedilol at a small starting dose. We were often not able to measure the plasma concentration of carvedilol in such patients over a period of 24 h by fluorometric detection. As we showed by our new method, the plasma concentration of carvedilol in such patients was as low as 0.10 ng/ml. There is no currently available method for measuring such a low plasma level of carvedilol. This is one of the reasons for the lack of information on pharmacokinetics of carvedilol in patients with CHF who have taken a low dose of the agent. ECD is accepted to be a high sensitive detector. However, there has been no report of measuring of carvedilol concentration by HPLC with ECD. We therefore developed a new method for measuring low concentrations of carvedilol, which was sufficient for clinical use.

It is also interesting to measure the concentration of carvedilol metabolites since these agents have anti-oxidant effect which is an added benefit of carvidilol administration. Concentrations of carvedilol metabolites have been measured by HPLC with fluorometric detection [15–18]. However, there has been no report of measurement of M21-aglycon in human plasma. M21-aglycon has a good electrical response in redox behavior. Its concentration can be measured by HPLC with ECD. And we attempted to identify the analyte in plasma by means of its responses to ECD and also to fluorometric detector. Apart from M21-aglycon, carvedilol and several metabolites were detected by both two of detector. M21-aglycon did not response to fluorometric detector. The peak at 21 min on the chromatogram of healthy volunteer detected by ECD (Fig. 5b)) was not detected by fluorometric detection, even after spiking (data were not shown). Therefore, we took the peak at 21 min as M21-aglycon.

We detected carvedilol as well as M21-aglycon in the healthy volunteer administered 30 mg of the agent, whose

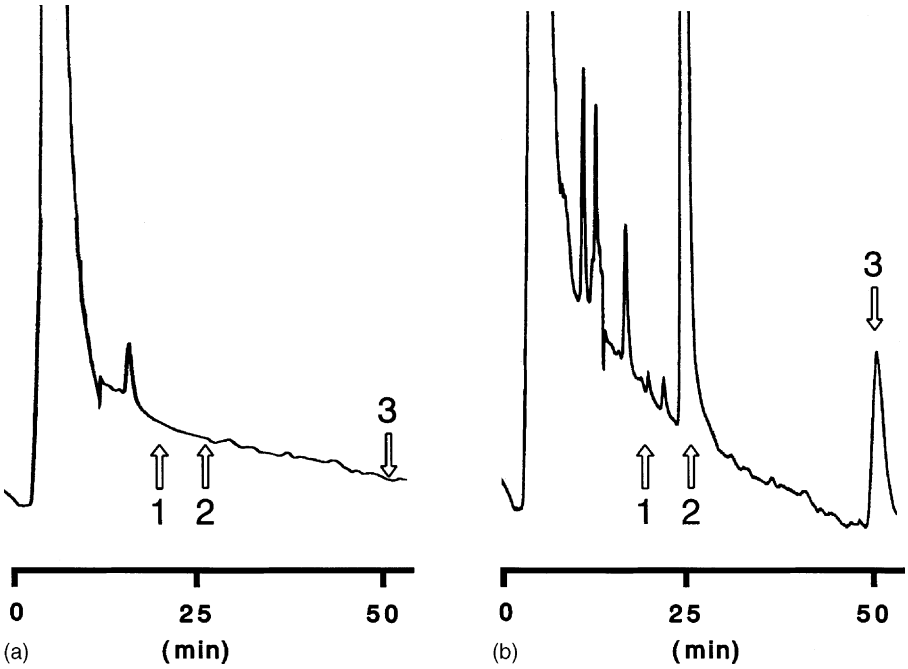

Fig. 5. Chromatograms of (a) blank drug-free plasma and (b) a sample from a healthy volunteer taken 2 h after oral administration of 30 mg of carvedilol. The concentrations of carvedilol and its M21-aglycon were 35.40 and 0.32 ng/ml, respectively. Peaks 1–3 represent carvedilol M21-aglycon (21.4 min), carvedilol (25.6 min) and internal standard (50.7 min), respectively.

level of M21-aglycon in plasma was 0.32 ng/ml. However, we only detected carvedillol in the three patients with CHF administered the agent at a dose of 2.5 mg, which was less than one tenth of the dose given to the healthy volunteer. We think that M21-aglycon was produced in the patients given low doses of the agent, though the peak of M21-aglycon was not identified this time on the chromatogram. The concentration of M21-aglycon might be able to be measured in such patients if the dose of the agent is clinically increased much more. The results of this study indicate that our method enables simultaneous measurements of levels of carvedilol and M21-aglycon in human plasma.

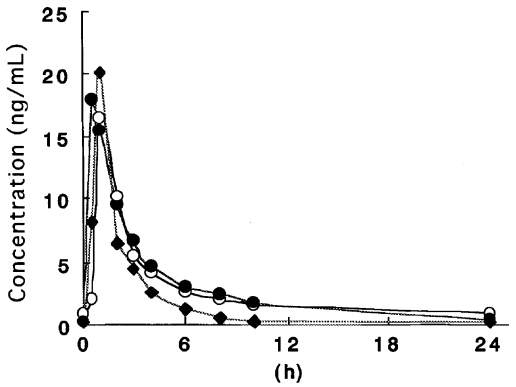

Fig. 6. Concentration–time curves of carvedilol in plasma following oral administration of 2.5 mg of carvedilol to the patients with CHF. Carvedilol was detected over a period of 24 h after administration.

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