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Analysis of carvedilol in human plasma using hydrophilic interaction liquid chromatography with tandem mass spectrometry

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Abstract

A rapid, sensitive and selective method for the determination of carvedilol in human plasma was developed using hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC–MS/MS). Carvedilol and cisapride (internal standard) were extracted from human plasma with methyl *tert*-butyl ether at basic pH and analyzed on an Atlantis HILIC Silica column with the mobile phase of acetonitrile–ammonium formate (50 mM, pH 4.5) (90:10, v/v). The analytes were detected using an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curve was linear ($r = 0.9998$) over the concentration range of 0.1–200 ng/ml. The lower limit of quantification for carvedilol was 0.1 ng/ml using 50 μl plasma sample. The coefficient of variation and relative error for intra- and inter-assay at four QC levels were 1.6–4.5% and −6.4 to 4.8%, respectively. The absolute and relative matrix effect for carvedilol and cisapride were practically absent. The extraction recoveries of carvedilol and cisapride were 81.6 and 85.2%, respectively. This method was successfully applied to the bioequivalence study of carvedilol in humans.

Keywords: Carvedilol; Human plasma; HILIC–MS/MS

1. Introduction

Carvedilol, 1-(9H-carbazol-4-yloxy)-3-[2-(2-methoxy-phenoxy)-ethylamino]-propan-2-ol, is an anti-hypertensive agent with non-selective β-and α_1-adrenergic receptor blocking activities approved for the treatment of congestive heart failure. Carvediol has been determined in biological fluids using high-performance liquid chromatography (HPLC) with fluorescence [1–7], electrochemical detection [8] or mass spectrometry (MS) [9–11], capillary electrophoresis with UV detection [12] and gas chromatography (GC) with MS detection [13]. Reverse-phase (RP)-HPLC methods were described for the analysis of carvedilol racemate [1,3–5,8,9] or carvedilol enantiomers using chiral derivatization [2,10]. HPLC methods using a chiral stationary phase have been reported for the analysis of carvedilol enantiomers [6,7].

The clean-up procedures for the extraction of carvedilol from biological matrix consist of protein precipitation [5,6,10,12], solid-phase extraction (SPE) [2,3,9], liquid–liquid extraction (LLE) [6,7,11–13], combinations of protein precipitation with SPE [4] or combinations of LLE with back-extraction [1,8]. Those methods use a large amount of biological samples (0.15–1.0 ml plasma or 2–5 ml urine samples) in order to obtain the high sensitivity or include time-consuming extraction procedures and/or relatively long run time.

Hydrophilic interaction liquid chromatography (HILIC) operated with bare silica and low aqueous/high organic mobile phase is a valuable tool in the quantitative analysis of the polar compounds in biological samples [14–22]. The higher organic content in the mobile phase of HILIC resulted in the sensitivity improvement and less matrix effect compared to RP–HPLC [14–16]. In this study, the use of HILIC–MS/MS on a silica column with high organic/low aqueous mobile phase is presented to analyze the polar carvedilol in human plasma. The rapid, robust and sensitive HILIC–MS/MS method using LLE with methyl *tert*-butyl ether was validated for the quantitative analysis of

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carvedilol using 50 μl human plasma and the present method has been successfully applied to the bioequivalence study of carvedilol in humans.

2. Experimental

2.1. Materials

Carvedilol and cisapride (internal standard) were the gifts from Dong-A Pharm. Co. Ltd. (Yongin, Korea). Acetonitrile and methyl *tert*-butyl ether (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of carvedilol and cisapride (1 mg/ml) were prepared in acetonitrile. Working standard solutions of carvedilol were prepared by diluting each primary solution with acetonitrile. The working solution for internal standard (25 ng/ml) was prepared by diluting an aliquot of stock solution with acetonitrile. All carvedilol and cisapride solutions were stored at 4 °C in polypropylene bottles in the dark when not in use.

Human plasma calibration standards of carvedilol (0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100 and 200 ng/ml) were prepared by spiking appropriate amount of the working standard solutions into a pool of 10 lots of drug-free human plasma. Quality control (QC) samples at 0.1, 0.6, 60.0 and 140 ng/ml were prepared in bulk by adding 100 μl of the appropriate working standard solutions (3.0, 18.0, 1800 and 4200 ng/ml) to drug-free human plasma (2900 μl). The QC samples were aliquoted (50 μl) into polypropylene tubes and stored at −20 °C until analysis.

2.3. Sample preparation

50 μl of blank plasma, calibration standards and QC samples were mixed with 10 μl of internal standard working solution and 300 μl of 0.5 M phosphate buffer (pH 9.0). The samples were extracted with 1000 μl of methyl *tert*-butyl ether in 1.5 ml-polypropylene tubes by vortex-mixing for 2 min at high speed and centrifuged at 5000 × *g* for 5 min at 4 °C. The organic layer (900 μl) was pipette transferred and evaporated to dryness under nitrogen at 35 °C. The residues were dissolved in 40 μl of acetonitrile by vortex-mixing for 2 min, transferred to injection vials, and 10 μl aliquots were injected for LC–MS/MS analysis.

2.4. LC–MS/MS analysis

The chromatographic system used for LC–MS/MS analysis consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on an Atlantis HILIC Silica column (5 μm, 3 mm i.d. × 50 mm, Waters Co., Milford, MA, USA) using a mixture of acetonitrile–ammonium formate (50 mM, pH 4.5) (90:10, v/v) at a flow rate of 0.5 ml/min. The column and autosampler tray were maintained at 40 °C and 4 °C, respectively. The analytical run time was 2.5 min. The eluent was introduced directly into the turbo ionspray source of a tandem quadrupole mass spectrometer (API 2000, Applied Bosystems/MDS SCIEX, Foster City, CA, USA). The turbo ionspray source was operated with typical settings as follows: ionization mode, positive; curtain gas, 35 psi; nebulizer gas, 50 psi; turbo gas, 65 psi; ionspray voltage, 5500 V; temperature, 380 °C. The molecular ions of carvedilol and cisapride were formed using the declustering potentials of 45 V and 50 V, respectively, and their molecular ions were fragmented at collision energy of 36 V and 29 V by collision-activated dissociation with nitrogen as the collision gas at a pressure setting of 7 on the instrument. Multiple reaction monitoring (MRM) mode was employed for the quantification: *m/z* 407.2 → 99.9 for carvedilol and *m/z* 466.1 → 183.8 for cisapride. Peak areas for all components were automatically integrated using Analyst software version 1.4 (Applied Biosystems/MDS SCIEX).

2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 0.1, 0.6, 60.0 and 140 ng/ml were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision, respectively. The selectivity was evaluated by analyzing blank plasma samples obtained from 30 different sources.

The absolute and relative matrix effect and recoveries of carvedilol and cisapride were assessed by analyzing three sets of standards at four concentrations (0.1, 0.6, 60.0 and 140 ng/ml) according to the approach of Matuszewski et al. [23]. The absolute matrix effect for carvedilol and cisapride was assessed by comparing mean peak areas of an analyte at four concentrations spiked after extraction into plasma extracts originating from five different lots (set 2) to mean peak areas for neat solutions of the analytes in acetonitrile (set 1). The variability in the peak areas of the analyte spiked post-extraction into five different plasma extracts (set 2) expressed as CVs (%), was considered as a measure of the relative matrix effect. Extraction recoveries of carvedilol were determined by comparing mean peak areas of analyte spiked before extraction into the same five different sources as set 2 (set 3) with those of the analyte spiked post-extraction into different blank plasma lots at four concentrations (set 2).

To assess post-preparative stability, six replicates of QC samples at each of the low and high concentrations (0.6 and 140 ng/ml, respectively) were processed and stored under autosampler conditions for 24 h before analysis.

2.6. Application

The developed HILIC–MS/MS method was used in a bioequivalence study after an oral administration of carvedilol to humans. Ten healthy male volunteers, fasted for 12 h, received a single oral dose of carvedilol (25 mg tablet) with 200 ml of water. Blood samples (2 ml) were withdrawn from the forearm vein at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h post-dosing, transferred to Vacutainer™ plasma glass tubes (sodium heparin, BD, NJ, USA) and centrifuged. Following centrifugation (3000 × *g*, 20 min, 4 °C), plasma samples were transferred to polypropylene tubes and stored at −20 °C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. The peak concentration (C_{max}) and the time to peak concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time plot for carvedilol. Area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 24 h. The protocol was approved by an institutional review board at Research Institute for Pharmaceutical Sciences, Sungkyunkwan University, Suwon, Korea and informed consent was obtained from the subjects after explaining the nature and purpose details of the study in accordance with Korean Guideline for Bioequivalence Test.

3. Results and discussion

3.1. HILIC–MS/MS

The electrospray ionization of carvedilol and cisapride produced the abundant protonated molecules ($[MH]^+$) at *m/z* 407.2 and 466.1, respectively under positive ionization conditions, without any evidence of fragmentation and adduct formation. $[MH]^+$ from carvedilol and cisapride were selected as the precursor ion and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 1). The fragment ions at *m/z* 99.9 (the loss of 9H-carbazol-4-yloxy and 2-methoxy-phenoxy group) and *m/z* 183.8 (5-chloro-4-amino-2-methoxy-phenyl-ketone) was produced as the prominent product ions for carvedilol and cisapride, respectively. The quantification of the analytes was performed using the MRM mode due to the high selectivity and sensitivity of MRM data acquisitions: *m/z* 407.2 → 99.9 for carvedilol and *m/z* 466.1 → 183.8 for cisapride.

HILIC–MS/MS methods operated with the silica column and low aqueous-high organic mobile phase have been proved to be ideal for the analysis of polar compounds in biological fluids [14–22]. Because increasing the content of water, a stronger elution solvent in HILIC, in the mobile phase decreased the retention of carvedilol and cisapride, a primary retention mechanism for carvedilol and cisapride may be also the hydrophilic interaction between the analytes and the silica stationary phase [14–16]. The higher organic content in the mobile phase of HILIC resulted in the sensitivity improvement *via* enhancement of ionization yield. Because of the higher sensitivity of HILIC–MS/MS method compared to that of RPLC–MS/MS, the plasma sample volume (50 μl) used in this study was smaller than that (200 μl) in RPLC–MS/MS of Borges et al. [11] to obtain the same LLOQ (0.1 ng/ml).

No interference at the retention times of carvedilol (1.4 min) and cisapride (1.3 min) was observed in any of the 30 different lots screened as shown in representative chromatogram of the extracted blank plasma sample, confirming the selectivity of the present method (Fig. 2a). The retention times of carvedilol and cisapride were reproducible throughout the experiment and no column deterioration was observed after analysis of 600 human plasma samples.

3.2. Method validation

This method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [24]. Calibration curves were obtained over the concentration range of 0.1–200 ng/ml of carvedilol in plasma. Linear regression analysis with a weighting of 1/concentration gave the optimum accuracy of the corresponding calculated concentrations at each

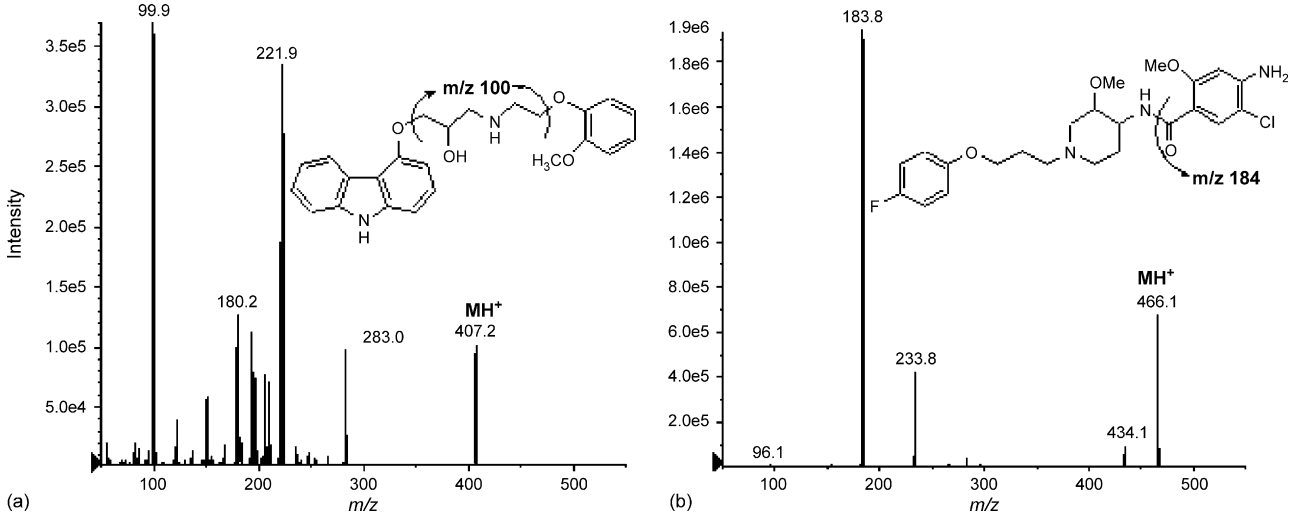

Fig. 1. Product ion mass spectra of (a) carvedilol and (b) cisapride (internal standard).

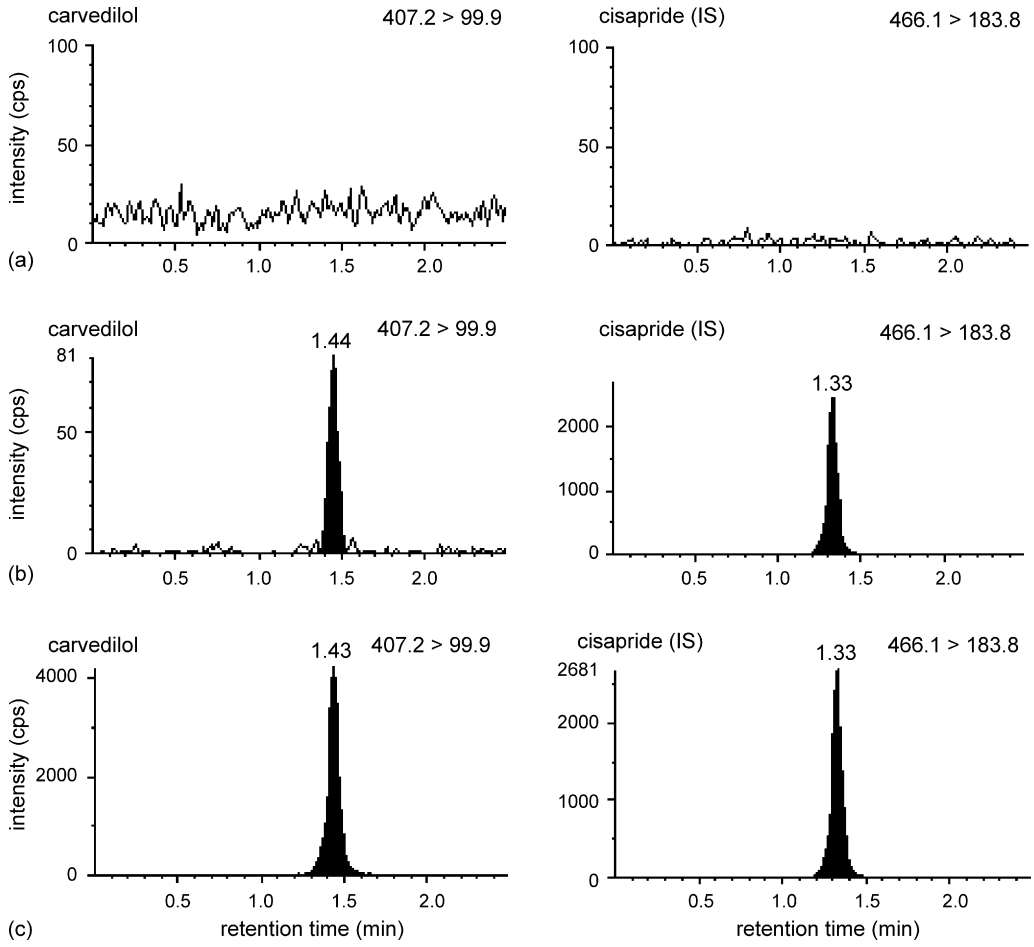

Fig. 2. MRM LC/MS/MS chromatograms of (a) a blank human plasma, (b) human plasma sample spiked with 0.1 ng/ml of carvedilol and (c) a plasma sample obtained 2 h after oral administration of carvedilol (25 mg) to a male volunteer.

Table 1
Calculated concentrations of carvedilol in calibration standards prepared in human plasma ($n = 9$)

	Theoretical concentration (ng/ml)								Slope	Intercept	R
	0.100	0.500	1.00	5.00	10.0	50.0	100	200			
Mean	0.101	0.474	1.00	5.14	9.84	48.9	99.8	198	0.0431	0.0021	0.9998
CV (%)	6.4	2.3	8.4	2.9	5.1	3.6	6.2	3.8	5.4		
RE (%)	1.0	−5.2	0.0	2.8	−1.6	−2.2	−0.2	−1.0			

level (Table 1). The low CV value for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-day precision and accuracy data for QC samples containing carvedilol. Both intra-and inter-assay CV values ranged from 1.6 to 4.5% at four QC levels. The intra- and inter-assay RE values for carvedilol were −6.4 to 4.8% at four QC levels. These results indicated that the present method has an acceptable accuracy and precision. The lower limit of quantitation (LLOQ) was set at 0.1 ng/ml for carvedilol using 50 μl of human plasma. Representative chromatogram of an LLOQ is shown in Fig. 2b and the signal-to-noise ratio for carvedilol is about 15 at 0.1 ng/ml. The limit of detection (LOD) was at 0.05 ng/ml for carvedilol.

Table 2
Precision and accuracy of carvedilol in quality control samples

	Intra-day ($n = 6$)				Inter-day ($n = 3$)			
QC (ng/ml)	0.100	0.600	60.0	140	0.100	0.600	60.0	140
Mean (ng/ml)	0.102	0.629	57.9	131	0.099	0.594	58.3	136
CV (%)	4.5	3.3	2.2	1.6	2.7	2.4	1.6	2.5
RE (%)	2.0	4.8	−3.5	−6.4	−1.0	−1.0	−2.8	−2.9

Table 3
Matrix effect and recovery data for carvedilol and cisapride (I.S.) in five different lots of human plasma.

Nominal concentration (ng/ml)	Mean peak area[a]						Matrix effect[b] (%)		Recovery[c] (%)	
	Carvedilol			Cisapride			Carvedilol	I.S.	Carvedilol	I.S.
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3				
0.100	587	576	447	15286	15351	13306	98.1	100.4	79.1	86.7
0.600	3075	3011	2401	15280	15334	12966	97.9	100.4	79.5	84.6
60.0	29013	28738	22478	15182	14963	12702	99.1	98.6	83.6	84.9
140	65769	65507	52494	14816	14809	12558	99.6	100.0	84.1	84.8
Mean							98.7	99.8	81.6	85.2

[a] In arbitary units, $n = 5$.
[b] Matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction (set 2) to the mean peak area of same analyte standards (set 1) multiplied by 100.
[c] Recovery calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked post-extraction (set 2) multiplied by 100.

The mean absolute matrix effect, the ratio of mean peak areas of set 2 to those of set 1 multiplied by 100 was 98.7% and 99.8% for carvedilol and cisapride, respectively (Table 3). A value of 100% indicates that the response in the solvent and in the plasma extracts were the same and no absolute matrix effect was observed. A value of <100% indicates an ionization suppression and a value of >100% indicates an ionization enhancement. These data indicate the absolute matrix effect for carvedilol and cisapride was practically absent.

The assessment of a relative matrix effect was made based on direct comparison of the peak areas of carvedilol and cisapride spiked post-extraction into extracts originating from five different sources of human plasma (set 2). The CVs of determination of set 2 at different concentrations varied from 4.1 to 8.7% for carvedilol and 3.3 to 4.9% for cisapride (Table 4). This variability seemed to be comparable to the precision of determination of standards injected directly in acetonitrile (set 1) (2.5–5.4% for carvedilol and 1.5–4.8% for cisapride, Table 4). These data confirm that the relative matrix effect for carvedilol and cisapride was practically absent. The CV of the ratio of carvedilol/cisapride for samples spiked post-extraction into extracts from five different lots of plasma varied from 3.4 to 5.7% at different concentrations and was similar to the CV of the ratio of carvedilol/cisapride injected directly in acetonitrile (2.5–5.6%, set 1 in Table 4), confirming that the absolute and relative matrix effects for ratio of carvedilol and cisapride have practically no effect on the determination of carvedilol spiked into five different lots of human plasma.

As shown in Table 3, the overall extraction recovery of carvedilol was 81.6%, which was consistent at four concentration levels. The extraction recovery of cisapride was 85.2%. The LLE with methyl *tert*-butyl ether at basic pH has been successfully applied to the extraction of carvedilol from human plasma.

Extracted QC samples were stable when stored at 4 °C for 24 h prior to injection, with <5% difference from theoretical concentration. Borges et al. [11] demonstrated the stability of carvedilol in human plasma samples through the stability tests including three freeze/thaw cycles, short-term (10 h) room temperature and long-term stability at −20 °C for 58 days.

3.3. Application study

This method has been successfully applied to the bioanalysis of 600 plasma samples in bioequivalence study of carvedilol. Representative chromatograms of the extract of a plasma sample obtained 2 h after oral dosing of carvedilol (25 mg) to human are shown in Fig. 2c. Fig. 3 shows mean plasma concentration profiles of carvedilol obtained after a single oral dosing of carvedilol (25 mg) to 10 healthy male volunteers. C_{max}, T_{max}

Table 4
Precision[a] (CV, %) of determination of peak areas of carvedilol and cisapride (internal standard), and peak area ratios (carvedilol/cisapride) in sets 1[b], 2[c] and 3[d]

Nominal concentration (ng/ml)	Precision (CV, %)								
	Peak area of carvedilol			Peak area of cisapride			Peak area ratio		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
0.100	3.8	8.7	8.3	4.8	4.9	4.2	2.5	5.7	9.6
0.60	5.4	4.1	7.4	1.5	3.3	4.5	5.6	3.5	6.6
60.0	3.4	7.0	6.6	3.7	4.7	5.6	3.9	3.7	7.7
140	2.5	4.3	5.5	4.8	4.5	5.0	4.6	3.4	5.9

[a] $n = 5$.
[b] Carvedilol and cisapride standards in acetonitrile.
[c] Carvedilol and cisapride spiked after extraction into extracts from five different plasma lots.
[d] Carvedilol and cisapride spiked before extraction into extracts from five different plasma lots.

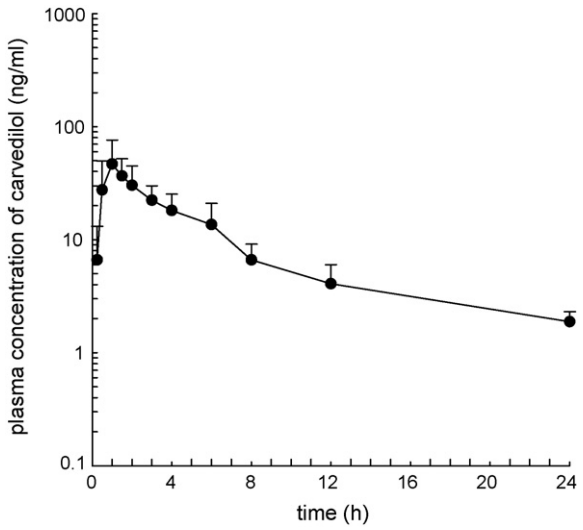

Fig. 3. Mean plasma concentration–time plot of carvedilol after a single oral dose of carvedilol (25 mg tablet) to 10 male volunteers. Each point represents the mean ± S.D.

and AUC of carvedilol were 47.8 ± 28.6 ng/ml, 1.1 ± 0.2 h and 238.1 ± 85.1 ng h/ml, respectively.

4. Conclusion

A rapid, sensitive and reliable HILIC–MS/MS method for the determination of carvedilol in human plasma has been successfully developed and validated using one-step liquid–liquid extraction as sample preparation procedure. This assay method demonstrated acceptable sensitivity (LLOQ: 0.1 ng/ml), precision, accuracy, selectivity, recovery and stability, and less absolute and relative matrix effect. The validated method was successfully applied to assay human plasma samples from the bioequivalence study of carvedilol.

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