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Original Article

A simple LC-MS/MS method for pharmacokinetic study of carvedilol and 4′-hydroxyphenyl carvedilol at a low dose

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Abstract

Background and purpose: The study was aimed at validating a simple, rapid, and low-cost LC-MS/MS method for carvedilol and 4′-hydroxyphenyl carvedilol assay in human plasma. The validated method was applied to investigate the pharmacokinetics after a low dose of 6.25 mg. carvedilol.
Experimental approach: In this study, the plasma was extracted by liquid-liquid extraction and evaporated the organic layer to dryness, then both analytes in the residue were reconstituted and detected by LC-MS/MS. The method was validated following the guideline on bioanalytical method validation. Thirty-one healthy volunteers participated in the pharmacokinetic study. After 10 h of fasting, each volunteer received one tablet of 6.25 mg carvedilol orally. Blood samples were collected at 16 prescheduled time points. The plasma samples were analyzed for pharmacokinetics.
Findings/Results: The method was linear over a range of 0.050-50.049 ng/mL for carvedilol and 0.050-10.017 ng/mL for 4′-hydroxyphenyl carvedilol. Crucial validated results reached the requirements of selectivity, accuracy, precision, and stability. Pharmacokinetics of carvedilol and 4′-hydroxyphenyl carvedilol were evaluated which showed C_{max} at 21.26 ± 9.23 and 2.42 ± 2.07 ng/mL; $AUC_{0\text{-}t}$ 66.95 ± 29.45 and 5.93 ± 3.51 ng.h/mL; $AUC_{0\text{-}inf}$ 68.54 ± 30.11 and 6.78 ± 3.49 ng.h/mL; and $T_{1/2}$ 6.30 ± 1.95 and 6.31 ± 6.45 h, respectively.
Conclusion and implications: The validated method was able to detect and quantify both analytes in plasma samples and can be applied to the pharmacokinetic study of carvedilol and 4′-hydroxyphenyl carvedilol after receiving carvedilol at 6.25 mg orally.

Keywords: Carvedilol; 4′-Hydroxyphenyl carvedilol; Pharmacokinetics; Tandem mass spectrometry.

INTRODUCTION

Carvedilol is a β- and α_1-adrenoceptor antagonist that is used to treat hypertension, angina pectoris, and chronic heart failure (1,2). Its β- and α_1 blocking activities cause peripheral vasodilation and reduce total peripheral resistance (1). Carvedilol is rapidly absorbed after oral administration with time to peak plasma concentration at approximately 1 to 2 h (3-5). The increasing peak plasma concentration is related to the administered dosage (3,6). It is highly lipophilic and ≥ 95% bound to plasma proteins (4). Its volume of distribution is about 1.5 to 2 L/kg (4,7). Due to the substantial first-pass metabolism, the absolute bioavailability is approximately 25% (4). It is rapidly and extensively metabolized in which unchanged form is found in the urine at less than 2% of the administered dose (8,9). The elimination half-life ranges from approximately 6 to 8 h (4,6).

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It is metabolized primarily by aromatic ring oxidation and glucuronidation (9). Three active metabolites that have been identified in humans include 4′-hydroxyphenyl carvedilol, 5′-hydroxyphenyl carvedilol (by CYP2D6 enzyme), and *O*-desmethyl carvedilol (by CYP2D6 and CYP2C9 enzymes) (10). 4′-hydroxyphenyl carvedilol and *O*-desmethyl carvedilol have approximately 13 and 2.5 times more β–adrenoceptor activity potent of than that of carvedilol, respectively (11). However, all of the metabolites possess weaker vasodilating activity (12) and their plasma concentrations are about 10-fold lower than that of carvedilol (12,13). Therefore, only 4′-hydroxyphenyl carvedilol which has high β-adrenoceptor potency may contribute to the activity (12).

In previous studies, pharmacokinetics in humans after oral intake in various dosage forms of carvedilol was reported. Patel *et al.* studied healthy subjects who were administered 12.5 mg carvedilol under fed condition and reported pharmacokinetics of carvedilol and 4′-hydroxyphenyl carvedilol by using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for assay (14). From the study of Carmo Borges *et al.* (15) and Kim *et al.* (16) with LC-MS/MS, the pharmacokinetics of carvedilol in healthy volunteers following administration of 25 mg carvedilol were reported. However, the pharmacokinetics of carvedilol and 4′-hydroxyphenyl carvedilol, after oral administration of a low dose of carvedilol at 6.25 mg have not been reported. Carvedilol is a racemic mixture of the S-(-) and R-(+) carvedilol enantiomers. Both enantiomers have α_1-receptor blocking activity, while the S enantiomer also has non-selective β-adrenoceptor blocking activity (2). However, a racemic mixture of S-(-) and R-(+) enantiomers are used in clinical therapeutic and in this study, the pharmacokinetics of carvedilol and 4′-hydroxyphenyl carvedilol were investigated as a racemic mixture of both enantiomers. This study aimed at modification and validation of a simple, rapid, low-cost, and reproducible assay by using LC-MS/MS for the measurements of both analytes and its application to a pharmacokinetic study in healthy volunteers when receiving a single oral dose of 6.25 mg carvedilol.

MATERIALS AND METHODS

Drug and chemicals

The product of carvedilol was DILATREND® (6.25 mg tablet) from F.Hoffmann-La Roche Ltd, Basel, Switzerland by Roche S.p.A. Milan production site Segrate, Italy. Carvedilol (purity 99.7%) and propranolol (purity 99.9%) were reference standards (USP). 4′-Hydroxyphenyl carvedilol (purity 98.5%) was a reference standard (TLC Pharmaceutical Standards Ltd). The product and all reference standards were supplied by Berlin Pharmaceutical Industry Co., Ltd., Thailand. Acetonitrile, methanol, tert-butyl methyl ether and formic acid were from Merck, Germany. Ammonium formate was from Sigma-Aldrich, Germany.

Chromatographic conditions

The carvedilol, 4′-hydroxyphenyl carvedilol, and propranolol (internal standard, IS) were assayed by LC-MS/MS (Shimadzu, Japan). The mass spectrometer with electrospray ionization was operated in the positive ionization mode to monitor protonated precursor → production transitions of *m/z* 407.10 → 100.10 for carvedilol, *m/z* 423.10 → 222.00 for 4′-hydroxyphenyl carvedilol and *m/z* 260.10 → 116.20 for IS. The chromatographic analysis was separated on the BDS hypersil column, (C18, 5 µm particle size and 2.1 × 150 mm). The mobile phase was a mixture of acetonitrile and 2 mM of ammonium formate (pH 3.0, adjusted by formic acid) in the ratio of 40/60 to 95/5 (v/v), at a flow rate of 0.4 mL/min. The autosampler and column oven were maintained at 4 °C and 40 °C, respectively.

Calibration curve and quality control samples

Stock solutions of each analyte were accurately weighed and dissolved in methanol to give the concentration at 1.0 mg/mL. The stock solution of carvedilol was diluted with water as the serial working solution and spiked into the blank plasma to set eight concentrations for calibration curve at 0.050, 0.100, 0.500, 2.502, 5.005, 10.010, 25.025, and 50.049 ng/mL and quality control (QC) samples at 0.050 ng/mL (lower limit of quantification, LLOQ), 0.15 ng/mL (low QC,

LQC), 15 ng/mL (medium QC, MQC) and 40ng/mL (high QC, HQC). Working solutions of 4′-hydroxyphenyl carvedilol were prepared by diluting the stock solution with methanol and made the calibration curve at 0.050, 0.101, 0.251, 0.503, 1.501, 4.003, 6.010, and 10.017 ng/mL and QC samples at 0.050 ng/mL (LLOQ), 0.150 ng/mL (LQC), 2.999 ng/mL (MQC), and 7.998 ng/mL (HQC). For IS, the compound was accurately weighed and diluted with methanol to prepare the stock solution (1.0 mg/mL) and working solution (301.798 ng/mL). Ten µL of each working solution of both analytes were spiked into 180 µL of blank plasma and added 301.798 ng/mL of IS to prepare calibration curve and QC samples. In the study, three calibration curves were constructed to test the linearity of the concentration range.

Sample preparation

Plasma samples (200 µL) and 10 µL of IS were pipetted into a test tube, then 2.5 mL of tert-butyl methyl ether was added. The mixture was vortex-mixed for 2 min. The organic layer was transferred into another test tube and evaporated by an evaporator. The residue was reconstituted with 150 µL of acetonitrile and 2 mM ammonium formate, pH 3 (50/50 v/v), and 5 µL was injected into the LC-MS/MS.

Method validation

The study was developed to set a particular method for the determination of both analytes in plasma samples. The method validation was performed by following the Bioanalytical Method Validation Guidance for Industry, U.S. FDA (17), and the Guideline Bioanalytical Method Validation, EMA (18).

Selectivity

Twelve sources of blank plasma were analyzed to evaluate for endogenous interference. The responses of the interfering peak at the retention time for the peak of carvedilol and 4′-hydroxyphenyl carvedilol should be less than 20% of the mean peak area of each analyte in the LLOQ and less than 5% of the mean peak area of IS in the LLOQ.

Linearity

Ten µL of each analyte were spiked into 180 µL of blank plasma and analyzed to construct the calibration curve. The peak area ratio of each analyte/IS versus the nominal concentration was plotted and fitted to linear regression with a weighting factor at $1/C^2$ (C = concentration). The back-calculated concentration of each level and deviation from the nominal value were evaluated. The acceptance criteria were ±15% deviation except for LLOQ, which was ±20%. In addition, a coefficient of determination (r^2) was required to be more than 0.99.

Accuracy and precision

Five replicates of LLOQ, LQC, MQC, and HQC were determined to assess intra-day and inter-day accuracy and precision. Both intra-day and inter-day acceptance criteria included the accuracy within ± 15% from the nominal value and the precision ± 15% CV except for LLOQ, which should be within 20%.

Recovery

The recovery was assessed by comparing the mean peak area of five replicates at LQC, MQC, and HQC of the analytes and IS from pre-extracted samples with the mean peak area of post-extracted samples. The recovery should be consistent, precise, and reproducible.

Matrix effect

The procedure was carried out by spiking the working solution of both analytes for preparing LQC and HQC and IS into six sources of extracted blank plasma. The matrix effect (MF) was assessed by comparing the mean peak area of three replicates at each concentration from extracted blank plasma with the mean peak area of the same concentration spiked in the solvent. IS-normalized MF was calculated from MF for each concentration of the analytes/MF for IS. The acceptance criteria for demonstrating the absence of matrix effect was IS-normalized MF within 0.80-1.20 and %CV ± 15.

Carry-over

Carry-over was assessed by injecting the blank samples after the upper limit of quantification (ULOQ). Carry-over in the blank sample following the ULOQ was not greater than 20% of the LLOQ and 5% of IS.

Dilution integrity

The solutions of both analytes were spiked into the blank plasma to obtain a concentration above ULOQ, then diluted with blank plasma for 2 and 4 folds. Five replicates of each concentration were determined and calculated by using the dilution factor. The acceptance criteria included accuracy within ± 15% and the precision ± 15% CV.

Hemolysis and lipemic effect

The responses of the interfering peak in hemolyzed and lipemic plasma at the retention time of both analytes should be less than 20% of the mean peak area of LLOQ and less than 5% of the mean peak area of IS in the LLOQ.

Stability

The solutions of both analytes were spiked into the blank plasma to prepare LQC and HQC. Three aliquots of each QC sample were analyzed at freshly prepared samples, then the QC samples were kept in various storage conditions to evaluate the stability of both analytes in plasma. The stability was evaluated by comparing the concentration of samples that were extracted immediately with the concentration of samples that were extracted after the three cycles of freeze at -70 °C for 24 h and thaw at room temperature (freeze-thaw stability), place on bench top at room temperature for 8 h (short term stability), storage in a freezer at -70 °C for 148 days (long term stability) and keeping in auto-sampler at 4 °C for 24 h (auto-sample stability). For stability in whole blood, the solutions of both analytes were spiked into whole blood to prepare LQC and HQC. Three replicates of each QC sample were determined and evaluated by comparing the samples that were extracted immediately with the samples that were extracted after being placed on the bench at room temperature for 30 min. The analytes were considered stable in plasma if the deviation was within ± 15%.

Pharmacokinetic study

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand, IRB No. 620/61 and conducted in accordance with the Declaration of Helsinki of the World Medical Association 2013. Informed consent was obtained from each volunteer before enrolling in the study. Thai healthy male and female volunteers with the age of 18-50 years old and body mass index of 18-25 kg/m^2 were enrolled in the study. The volunteers underwent screening tests and were confirmed as healthy by physical examination, vital signs, electrocardiogram, and clinical laboratory screenings. All volunteers abstained from other drugs and alcoholic preparation intakes two weeks prior to and throughout the study. Caffeine-containing beverages were prohibited 3 days prior to and throughout the study.

Each volunteer fasted for 10 h prior to the dosing. On the study day, each volunteer received one tablet of carvedilol (6.25 mg) orally with 250 mL water. Food was not permitted until 4 h after dosing. The blood samples were collected at the following time points: pre-dose, 0.33, 0.67, 1, 1.33, 1.67, 2, 2.5, 3, 3.5, 4, 6, 9, 12, 24, and 30 h after drug administration. The plasma samples were kept in a freezer at -70 °C until being analyzed. In each analytical run consisted of the blank sample, calibration curve, 2 sets of QC samples at least 3 levels, and plasma samples of volunteers. Acceptance criteria of the analytical run included the accuracy of calibration curve within ±15 from the nominal value, except the LLOQ for which it should be within ±20% and at least 67% of QC samples and at least 50% at each concentration level within ±15 accuracy from the nominal value.

Pharmacokinetic analysis

Pharmacokinetic parameters consisted of peak plasma concentration (C_{max}), time to peak plasma concentration (T_{max}), and area under the concentration versus time curve (AUC): AUC from 0 to 30 h (AUC_{0-t}) and AUC from 0 to infinity (AUC_{0-inf}), terminal elimination half-life ($T_{1/2}$), clearance, and volume of distribution (Vd). Phoenix WinNonlin software, version 8.1 was used to assess the pharmacokinetic parameters.

The C_{max} and T_{max} of each volunteer were calculated from the individual concentration versus time. The AUC was calculated by the linear-log trapezoidal method. The elimination rate constant (Kel) was estimated by log-linear least squares regression of the terminal part of the plasma concentration versus time curve. The $T_{1/2}$ was calculated by $T_{1/2}$ = ln2 /$K_{el.}$ (In = 0.693). The clearance was calculated from the equation of F*dose/$AUC_{0\text{-inf}}$ with the 25% bioavailability (F = bioavailability) as reported in the previous study (4). The Vd value was calculated from clearance divided by Kel.

RESULTS

Method validation

Selectivity, hemolysis, and lipemic effect

Normal blank plasma including hemolyzed and lipemic blank plasma was analyzed and showed any absence of endogenous interferences at the retention time of the peaks of carvedilol (4.1 min), 4′-hydroxyphenyl carvedilol (3.2 min), and IS (3.7 min.) that the results demonstrated good selectivity of the method. The chromatograms of blank plasma, blank plasma spiked with both analytes and IS, and plasma samples from volunteers collected after administration of 6.25 mg carvedilol are presented in Fig. 1.

Linearity

Calibration curve of carvedilol and 4′-hydroxyphenyl carvedilol showed linearity in the range of 0.050-50.049 ng/mL and 0.050-10.017 ng/mL, respectively and the correlation coefficients (R^2) of both analytes were greater than 0.9928. From three calibration curves, the back-calculated concentrations of carvedilol and 4′-hydroxyphenyl carvedilol in all levels were ranged from 88.000% - 112.889% and 91.633% - 113.0861%, respectively. The mean equation of the calibration curve was y = 0.063157x + 0.001947 (R^2 = 0.9970) and y = 0.090737x - 0.028465 (R^2 = 0.9956) for carvedilol and 4′-hydroxyphenyl carvedilol, respectively.

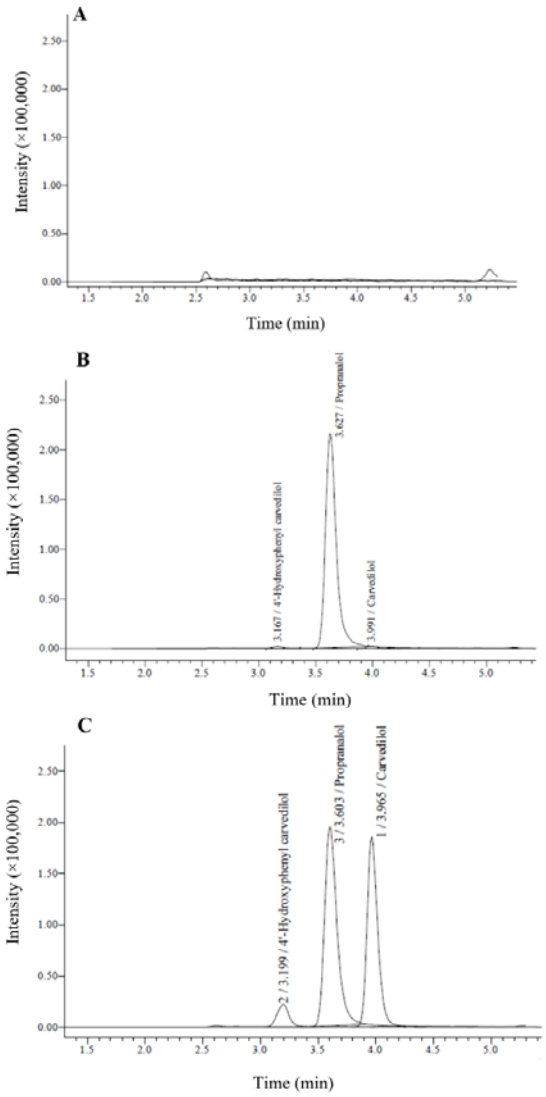

Fig. 1. Represent chromatogram of carvedilol, 4′-hydroxyphenyl carvedilol, and propranolol in (A) human blank plasma, (B) plasma at the lower limit of quantification with IS, and (C) volunteer sample after 1 h oral administration of 6.25 mg carvedilol with propranolol. Propranolol was used as an internal standard.

Accuracy and precision

Intra-day accuracy of carvedilol and 4′-hydroxyphenyl carvedilol was in the range of 91.200% - 106.049% and 88.792% - 105.600%, respectively while the precision (%CV) was less than 11.769% and 9.671%, respectively. For inter-day, the accuracy of carvedilol and 4′-hydroxyphenyl carvedilol was in the range of 98.006% - 102.782% and 91.569% - 110.667%, respectively while the precision was less than 10.697% and 9.633%, respectively. The results of intra-day and inter-day accuracy and precision are summarized in Table 1.

Table 1. Intra- and inter-day accuracy and precision of carvedilol and 4′-hydroxyphenyl carvedilol.

Analytes	QC level (ng/mL)	Intra-day (n = 5)			Inter-day (3 days)		
		Mean concentration (ng/mL)	Accuracy (%)	Precision, CV (%)	Mean concentration (ng/mL)	Accuracy (%)	Precision, CV (%)
Carvedilol	LLOQ (0.050)	0.046	91.200	11.769	0.051	102.667	8.998
	LQC (0.150)	0.149	99.333	8.096	0.150	99.778	10.697
	MQC (15.000)	15.907	106.049	2.455	15.417	102.782	6.959
	HQC (40.000)	38.618	96.545	2.386	39.202	98.006	4.018
4′-Hydroxy-phenyl carvedilol	LLOQ (0.050)	0.053	105.600	8.190	0.055	110.667	3.762
	LQC (0.150)	0.138	92.000	8.166	0.148	98.444	9.633
	MQC (2.999)	3.015	100.540	4.169	2.973	99.144	7.315
	HQC (7.998)	7.102	88.792	9.671	7.324	91.569	8.843

QC, Quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

Recovery

The mean recovery of carvedilol and 4/-hydroxyphenyl carvedilol were ranged from 80.760% - 88.415% and 72.424% - 100.589%, respectively while the precision (%CV) were ranged from 3.506% - 13.969% and 3.526% - 13.377%, respectively. The method showed consistent, precise, and reproducible extraction recovery.

Matrix effect

Mean matrix factors (±CV) at LQC and HQC were 0.859 (6.262%) and 0.975 (9.896%) for carvedilol and 1.149 (3.684%) and 0.845 (4.295%) for 4/-hydroxyphenyl carvedilol, respectively while mean IS-normalized MF (±CV) were 0.874 (5.001%), and 0.996 (8.340%) for carvedilol, and 1.169 (1.135%) and 0.864 (5.618%) for 4/-hydroxyphenyl carvedilol, respectively. The results were within the acceptance criteria.

Carry-over

An analyzing series of blank samples were continuously injected after ULOQ injection where the peak responses of carvedilol and 4/-hydroxyphenyl carvedilol were not detected in the blank plasma.

Dilution integrity

The samples were prepared at two times of HQC (80.000 ng/mL for carvedilol and 15.996 ng/mL for 4/-hydroxyphenyl carvedilol) and diluted with blank plasma. From the back calculating concentration by using the dilution factor, the accuracy (with the precision) of 2 and 4-fold dilution were 96.99% (5.041% CV) and 95.56% (6.422% CV) for carvedilol and 110.10% (2.896% CV), and 104.57% (10.099% CV) for 4/-hydroxyphenyl carvedilol, respectively.

Stability

The summarized results presented in Table 2 indicate that the deviation of carvedilol and 4/-hydroxyphenyl carvedilol after storage of the samples in various conditions compared with the concentration of samples that were extracted immediately was less than 15%. Therefore, both analytes were stable in the samples after freezing at -70 °C and complete thaw at room temperature for 3 cycles, placing on the benchtop at room temperature up to 8 h, placing in an auto-sampler of the instrument at 4 °C for 24 h, storing at -70 °C for long period until 148 days including separation of plasma from whole blood after blood collection for 30 min.

Table 2. The stability results of carvedilol and 4/-hydroxyphenyl carvedilol, n = 3.

Storage conditions	Analytes	QC Level	Mean concentration of freshly prepared samples (ng/mL)	Mean concentration of stability samples (ng/mL)	Deviation (%)
Freeze-thaw stability (3 cycles, -70 °C)	Carvedilol	LQC	0.148	0.147	-1.124
		HQC	37.498	42.722	13.931
	4/-Hydroxy phenyl	LQC	0.134	0.137	1.990
		HQC	7.076	6.849	-3.213
Short-term stability (8 h, room temperature)	Carvedilol	LQC	0.157	0.164	4.246
		HQC	45.022	42.671	-5.220
	4/-Hydroxy phenyl	LQC	0.150	0.157	5.122
		HQC	7.127	6.913	-3.003
Long-term stability (148 day, -70 °C)	Carvedilol	LQC	0.145	0.158	8.716
		HQC	39.135	39.854	1.836
	4/-Hydroxy phenyl	LQC	0.134	0.148	10.448
		HQC	7.076	7.538	6.519
Post-preparative stability in auto-sampler (24 h, 4 °C)	Carvedilol	LQC	0.150	0.160	6.652
		HQC	41.382	40.192	-2.876
	4/-hydroxy phenyl	LQC	0.151	0.149	-1.545
		HQC	8.869	8.290	-6.528
Stability in whole blood (30 min, room temperature)	carvedilol	LQC	0.140	0.155	10.556
		HQC	45.910	45.723	-0.408
	4/-hydroxy phenyl	LQC	0.156	0.160	2.308
		HQC	7.533	7.098	-5.770

QC, Quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

Application to the pharmacokinetic study

There were 31 participants (15 males and 16 females) in the study. The average age was 34.23 ± 8.21 years and the body mass index was 22.28 ± 1.83 kg/m^2. All volunteers were confirmed healthy according to the results of their physical examination, vital signs, electrocardiograms, and clinical laboratory screenings. The complete blood count, fasting blood sugar, blood urea nitrogen, serum creatinine, alkaline phosphatase, alanine transaminase, aspartate transaminase, total bilirubin, albumin, electrolytes were within the normal limit. Hepatitis Bs-antigen and anti-HIV were negative. Two adverse events, headache, and pharyngitis were reported during the study period. Headache was classified as probably related and pharyngitis was unrelated to the study. However, no serious adverse events were reported after the drug administration.

In all analytical runs, at least 67% of the back-calculated concentrations of the calibration curve with a minimum of six levels and QC samples both of carvedilol and 4′-hydroxyphenyl carvedilol were met the acceptance criteria of the accuracy within ±15 from the nominal value. All pharmacokinetic results of carvedilol and 4′-hydroxyphenyl carvedilol are presented in Table 3. The mean plasma concentrations of carvedilol and 4′-hydroxyphenyl carvedilol are displayed in Fig. 2.

Table 3. Pharmacokinetic parameters of carvedilol and 4′-hydroxyphenyl carvedilol after administration of a single 6.25 mg carvedilol orally.

Parameters	Carvedilol		4′-Hydroxyphenyl carvedilol	
	Mean geometric (SD)	**Range (min-max)**	**Mean geometric (SD)**	**Range (min-max)**
T_{max} (h)	0.78 (0.34)	0.33 - 2.00	0.79 (0.32)	0.33 - 2.00
C_{max} (ng/mL)	21.26 (9.23)	8.67 - 44.94	2.42 (2.07)	0.73 - 9.56
$AUC_{0\text{-}t}$ (ng.h/mL)	66.95 (29.45)	26.39 - 140.67	5.93 (3.06)	1.98 - 15.24
$AUC_{0\text{-}inf}$ (ng.h/mL)	68.54 (30.11)	27.16 - 143.94	6.78 (3.49)	2.23 - 18.13
$T_{1/2}$ (h)	6.30 (1.95)	2.82 - 12.04	6.31 (6.45)	2.34 – 24.34
Clearance (L/h)	22.79 (12.05)	10.86 - 57.54		
volume of distribution (L)	208.75 (126.78)	98.07 - 549.98		

T_{max}, Time to peak plasma concentration; C_{max}, peak plasma concentration; AUC, area under the curve.

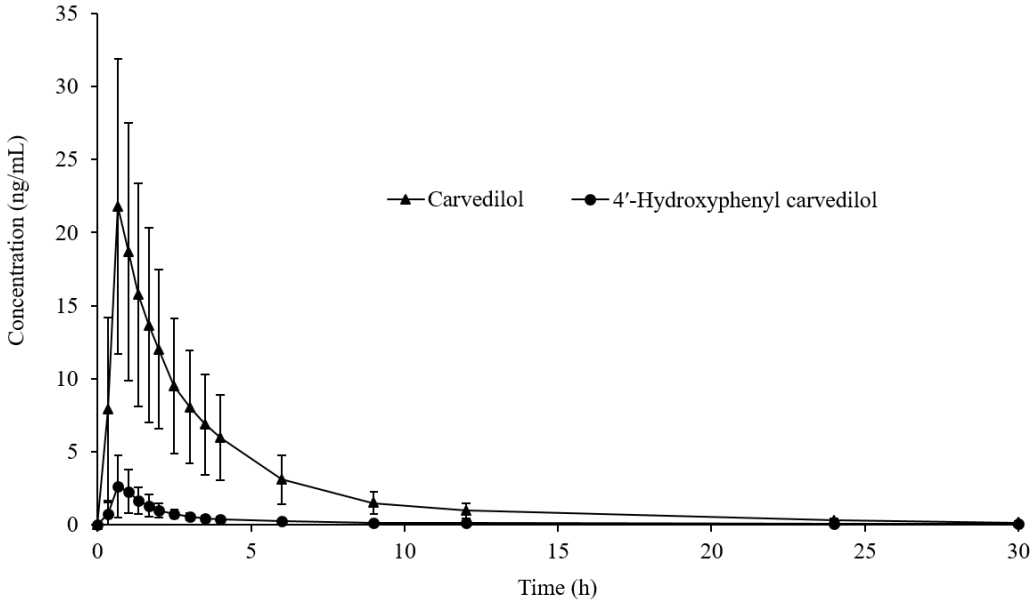

Fig. 2. Plasma concentration-time profiles following oral single carvedilol 6.25 mg tablet of carvedilol and 4′-hydroxyphenyl carvedilol. Data represent mean ± SD.

DISCUSSION

From the validated results, the present method showed good selectivity with no endogenous interference derived from red blood cells and lipid in the plasma that did not affect the analysis. The calibration curve showed good linearity with the coefficient of determination (R^2) close to 1 and high sensitivity with LLOQ at 0.05 ng/mL for both analytes. The method was accurate and precise demonstrated by intra-day and inter-day accuracy and precision test, as well as diluting the samples with blank plasma which did not affect the accuracy and precision. The residual of both analytes from the sample analyzed previously in the run were not observed as carry-over in the method. The stability test demonstrated no degradation of analytes during collection and storage of plasma samples in the various condition which covered the duration of the sample storage in this study. In particular, the results of the long-term stability test showed that plasma samples could be stored at -70 °C for up to 148 days. All of the validated results showed the method could analyze and quantify both analytes in the plasma samples and could be applied for pharmacokinetic study.

Some methods for the determination of carvedilol and 4′-hydroxyphenyl carvedilol in plasma have previously been published. Nardotto *et al.* (19) and Gangnus *et al.* (20) determined the LLOQ of carvedilol and 4′-hydroxyphenyl carvedilol, using LC-MS/MS, at 0.02 ng/mL and 0.024 ng/mL, respectively that showed high sensitivity. However, both previous assays were more complicated, took more turnaround time, and were extravagant compared to the present study. The extraction procedure of the present method took only 2 min and consumed less organic solvent. So, the method exhibited a very simple, rapid, and low-cost assay for both analytes. Huang *et al.* demonstrated a very simple and rapid UPLC-MS/MS but it had a poor sensitivity of LLOQ at 0.5 ng/mL for carvedilol and 0.1 ng/mL for 4′-hydroxyphenyl carvedilol (21). Also, in another study, by using LC-MS/MS, LLOQ of 4′-hydroxyphenyl carvedilol was detected to be 0.02 ng/mL while LLOQ of carvedilol was 0.20 ng/mL indicating poor sensitivity and longer retention time of both analytes relative to the results of the present study (22).

The results of this study revealed that after a single oral 6.25 mg carvedilol tablet, healthy Thai volunteers showed rapid absorption of carvedilol and rapid metabolism of carvedilol to 4′-hydroxyphenyl carvedilol with a T_{max} less than 1 h. T_{max} of carvedilol at 0.78 h was faster than T_{max} at 0.94 h after 12.5 mg dosing reported by Kim *et al.* (23), and 1.25 h after 25 mg by Cho *et al.* (24). Carvedilol at a low dose was rapidly absorbed compared to the larger doses. The $T_{1/2}$ of 6.31 h of this study was within the range of 6 to 8 h from the previous studies (4,6).

The C_{max} of carvedilol found in the present study was 21.26 ng/mL which was related to dose with the reported C_{max} of 39 ng/mL after administration of 12.5 mg, 75 ng/mL after 25 mg, and 161 ng/mL after 50 mg of carvedilol (6) and 107.7 ng/mL after 25 mg dosing (16). However, it was different from the C_{max} of 23.65 ng/mL following the 12.5 mg dose reported by Kim *et al* (23) and 35.8 ng/mL after 25 mg dose (24). Previous studies reported that CYP2D6 polymorphisms caused individual variations in the ability of carvedilol metabolism (25,26). Jung *et al.* reported the decrease in CYP2D6 activity influenced the carvedilol pharmacokinetics in healthy Korean volunteers (25). In addition, Honda *et al.* reported a significant increase of the carvedilol bioavailability in Japanese volunteers with a common allele, CYP2D6*10. These findings may indicate the effects of inter-individual variability of bioavailability and different ethnicities on the pharmacokinetics of carvedilol (26).

The plasma concentration of 4′-hydroxyphenyl carvedilol was about 10 times lower than that of carvedilol which was similar to the previous reports (12,13). Although the metabolites had higher potency of β-adrenoceptor blocking activity than carvedilol, they may be less effective or not greater than the carvedilol due to the low concentrations of these metabolites (12).

CONCLUSION

In conclusion, the current study fully validated the method for the determination of carvedilol and 4/-hydroxyphenyl carvedilol in human plasma and all the results met the requirements of bioanalytical method validation indicating a simple, rapid, and highly sensitive established method for quantification of both analytes. Therefore, the method was applied for pharmacokinetic study in healthy volunteers who received low doses of carvedilol orally at 6.25 mg under fasting conditions.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

W. Eiamart developed the method for sample analysis and data management; N. Prompila contributed to designing the protocol and interpreting the data; Y. Jumroen analyzed the plasma samples; N. Sayankuldilok was involved to assure and verify the data, P. Chariyavilaskul acted as a clinical investigator and conducted the clinical study, and S. Wittayalertpanya reviewed and edited the study protocol and the manuscript. The final version of the manuscript was approved by all authors.

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