Quantitative determination of carvedilol in human plasma by high-performance liquid chromatography using fluorescence detection

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Abstract

Background: Carvedilol is a pharmaceutical substance listed in the "Regulatory requirements of bioequivalence study reports for generic drugs containing APIs upon applying for marketing authorization". Therefore, a simple method for quantifying carvedilol in human plasma is desirable. **Objectives:** This study aims to develop an HPLC method for quantitation of carvedilol in human plasma. **Materials and method:** The blank human plasma was spiked with carvedilol standard. After optimizing the process, the method was validated according to the guidelines for the validation of bioanalytical methods of US-FDA and EMA. **Results:** Carvedilol and metoprolol as internal standard were extracted from plasma by protein precipitation technique with acetonitrile. Plasma samples were eluted through a Zorbax Eclipse XDB-C8 (5 µm; 4.6 x 150 mm) column with an isocratic mobile phase consisting of 0.1% trifluoroacetic acid in water, acetonitrile, and methanol (60:20:20; v/v/v). The analytical method met the criteria according to the US-FDA and EMA guidelines for the bioanalytical method validation. **Conclusion:** The method can be applied to determine carvedilol in biological fluid for pharmacokinetic research and bioequivalence assessment.

Keywords: *carvedilol, human plasma, HPLC.*

1. INTRODUCTION

In recent years, cardiovascular diseases have been increasingly recognized as one of the leading causes of morbidity and mortality in developed countries. In most cases, individuals with cardiovascular diseases may experience symptoms such as chest pain and fatigue, while in many instances, individuals may remain asymptomatic until they experience a heart attack [1]. Therefore, early detection and treatment of these conditions are of paramount importance. Carvedilol is a nonselective β receptor blocker with vasodilatory and antioxidant properties, which is clinically used to treat cardiovascular disorders such as mild to moderate hypertension or angina pectoris [2].

All over the world, several methods have been published for the estimation of carvedilol in rat plasma [3], human plasma [4-6], and human urine [7] using high-performance liquid chromatography (HPLC) coupled to a fluorescence detector [5], [6], [8], ultraviolet detector [7], [9], liquid chromatography coupled to tandem mass spectrometry[10], gas chromatography coupled to tandem mass spectrometry [11] and ultra performance liquid chromatography (UPLC) coupled to tandem mass spectrometry [3, 12]. However, these methods have several limitations, including complex sample preparation procedures, lengthy analysis times, and the use of hazardous solvents that pose risks to both human health and the environment. In methods involving mass spectrometry (MS) coupling, reports have shown high sensitivity and low limit of quantitation (LLOQ). The best LLOQ of 0.05 ng/mL was reached when using the UPLC-MS/MS method. However, these methods are costly and require specialized equipment, making them not suitable for all laboratories. Thus, in this paper, we present a simple, accurate, and cost-effective HPLC method using a fluorescence detector with a simple sample preparation for quantifying carvedilol in human plasma.

2. MATERIALS AND METHODS

2.1. Materials

Carvedilol (98%) and metoprolol tartrate (100.37%) were provided by Toronto Research Chemicals, Canada, and National Institute of Drug Quality Control, Vietnam, respectively. HPLC-grade acetonitrile, methanol, and all other analytical grade chemicals were purchased from Merck, Darmstadt, Germany. Ultrapure water was obtained from an ultrapure water system, AltoTOC UF, AVIDITY, UK. Human plasma was supplied by National Institute of Hematology and Blood Transfusion, Vietnam.

The HPLC system of Shimadzu, Japan consisted of a pump LC-20AD, an online degassing unit DGU-20A,

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an automatic sample injector SIL-20A, and a model RF-20A fluorescence detector. The results were analyzed by LC solution version 1.2 software. The elution was performed on a Zorbax Eclipse XDB-C8 column (5 µm; 4.6x150 mm). The mobile phase was a mixture containing 0.1% trifluoroacetic acid (TFA) in water, acetonitrile, and methanol (60:20:20, v/v), with a flow rate of 1 mL/min. The elution time was set for 20 min with the excitation/emission wavelength gradient program.

2.2. Methods

2.2.1. Preparation of standard solutions

Stock solutions of carvedilol and metoprolol of 1 mg/mL were prepared in methanol. These solutions were diluted with methanol to obtain working standard solutions of 1000 ng/mL.

2.2.2. Preparation of the sample solution

The human plasma was thawed at room temperature. Subsequently, 100 µL of metoprolol at a concentration of 1000 ng/mL, used as the internal standard (IS), and 1 mL of acetonitrile were added to a centrifuge tube containing 900 µL of plasma and vortexed for 2 min. After that, the mixture was centrifuged at 10000 rpm (Hermle Z326K, Germany) for 10 min. Then, the supernatant was filtered through a 0.45 µm nylon membrane filter to obtain the filtrate, and 20 µL of this solution was injected into the HPLC system.

2.2.3. Preparation of quality control sample solutions

Quality control samples (QCs) included the lower and upper limits of quantitation (LLOQ and ULOQ), as well as low, middle, and high-quality controls (LQC, MQC, HQC). These samples are plasma samples containing a carvedilol standard solution at concentrations of 2.5, 100, 7.5, 30, and 75 ng/mL, respectively, along with the internal standard solution at a final concentration of 100 ng/mL.

2.2.4. Method validation

The validation of the analytical method was performed according to the US-FDA 2018 [13] and EMA 2012 [14] guidelines through the following criteria: system suitability, calibration range, selectivity and specificity, carryover, sensitivity, accuracy and precision, recovery, stability, and dilution effects.

2.2.4.1. Calibration curves

The calibration curve consisted of plasma samples containing carvedilol standard solutions at concentrations of 2.5, 7.5, 10, 30, 75, and 100 ng/mL, along with the IS solution at concentration of 100 ng/mL. The correlation between the concentration of carvedilol on the X-axis and the peak area ratio of carvedilol and IS on the Y-axis was represented by a linear regression equation y = ax + b, where the correlation coefficient was greater than or equal to 0.98.

2.2.4.2. Selectivity and specificity

It was determined by comparing peaks in the chromatograms of blank plasma samples and plasma samples containing carvedilol and IS. The IS and carvedilol response in the blank and the average IS and carvedilol responses of the calibrators and QCs were also considered.

2.2.4.3. Cross validation

It was assessed by the impact of carryover on the accuracy of the study sample concentrations.

2.2.4.4. Dilution effects

The integrity of the dilution process should be monitored during validation by diluting QCs above the ULOQ with a similar matrix to bring them within the quantitation range. Additionally, the accuracy and precision of these diluted QCs must be demonstrated.

2.2.4.5. Accuracy and precision

The intra-day and inter-day accuracy and precision (A&P) were established with three independent A&P runs on three consecutive days. Each run consisted of four QC levels (LLOQ, LQC, MQC, HQC), and each QC sample was replicated six times. The concentrations were calculated from the calibration curve under the same analytical conditions.

2.2.4.6. Lower limit of quantitation

The lower limit of quantitation was defined as the lowest concentration at which the analyte response must be at least five times the analyte response of the blank. At this concentration, the accuracy must be within ± 20%, and the relative standard deviation must be less than 20%.

2.2.4.7. Recovery

Three QC samples (LQC, MQC, HQC) were prepared following the previously mentioned sample preparation procedure. To assess the recovery of carvedilol and the internal standard (IS), we compared their responses in the extracted QC samples to those in the non-extracted QC samples.

2.2.4.8. Stability

The stability of the analyte in the plasma samples was evaluated in the short-term and long-term periods when storing samples at room temperature for 6 hours and storage at –35 ± 5 °C for 30 days, respectively. The plasma samples were also frozen at –35 ± 5 °C to analyze the stability of carvedilol

after three freeze-thaw cycles. The stability of stock solutions was assessed at room temperature for 6 hours and at -20 °C for 30 days by comparing these concentrations with freshly prepared solutions on the first day of analysis.

3. RESULTS

3.1. Chromatographic condition

Following the investigation of C18 and C8 column systems, the Zorbax Eclipse XDB-C8 column (5 µm; 4.6 x 150 mm) was chosen for this study. After conducting experiments with various mobile phase systems at different ratios, the optimal mobile phase consisted of 0.1% TFA in water, acetonitrile, and methanol in volumetric proportions of 60:20:20. The gradient wavelength pairs were adjusted over time as follows: from 0.01 to 10 min using a wavelength of 276/296 nm; from 10.01 to 17 min using a wavelength of 240/330 nm; from 17.01 to 20 min using a wavelength of 276/296 nm for the peak detection of aesthetically pleasing derivatives of the active compound, achieving balance.

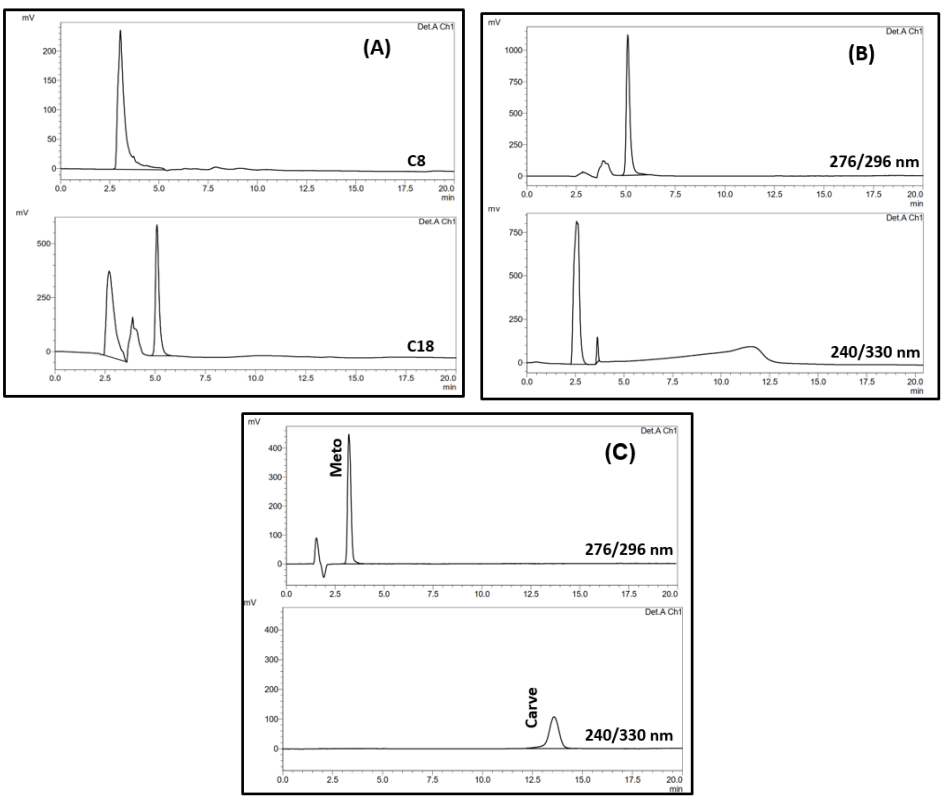

Figure 1. Spectra of the chromatographic condition of (A) the investigation of C18 and C8 column systems; (B) phosphate buffer pH 2, acetonitrile, and methanol in volumetric proportions of 60:20:20 on a C8 column; (C) 0.1% TFA in water, acetonitrile, and methanol in volumetric proportions of 60:20:20 on a C8 column

3.2. Method validation

For the system suitability test, all criteria (retention time, peak area, peak area ratio between carvedilol and IS, tailing factor, and resolution) meet the requirements according to the standards of the US-FDA and EMA. The results are presented in detail in Table 1.

Table 1. System suitability compared to criteria of the US-FDA and EMA guidelines

	Retention time	Peak area	Peak area ratio	Tailing factor (mean ± SD)	Resolution (mean ± SD)
Carvedilol	0.27	1.81	2.37	0.96 ± 0.01	4.61 ± 0.07
IS	0.94	0.85		1.15 ± 0.21	3.28 ± 0.12
Acceptance criteria	RSD* ≤ 3%			$0.8 \leq T_f \leq 1.5$	$R_s \geq 1.5$

*RSD: the relative standard deviation

The results in Figure 1 demonstrated that at the respective retention times of the IS and carvedilol peaks, no interfering peaks were observed in the chromatogram of the blank sample. Furthermore, the analyte response at the LLOQ was five times greater than the analyte response of the zero calibrator. Based on these observations, it can be concluded that this procedure exhibits high selectivity and specificity.

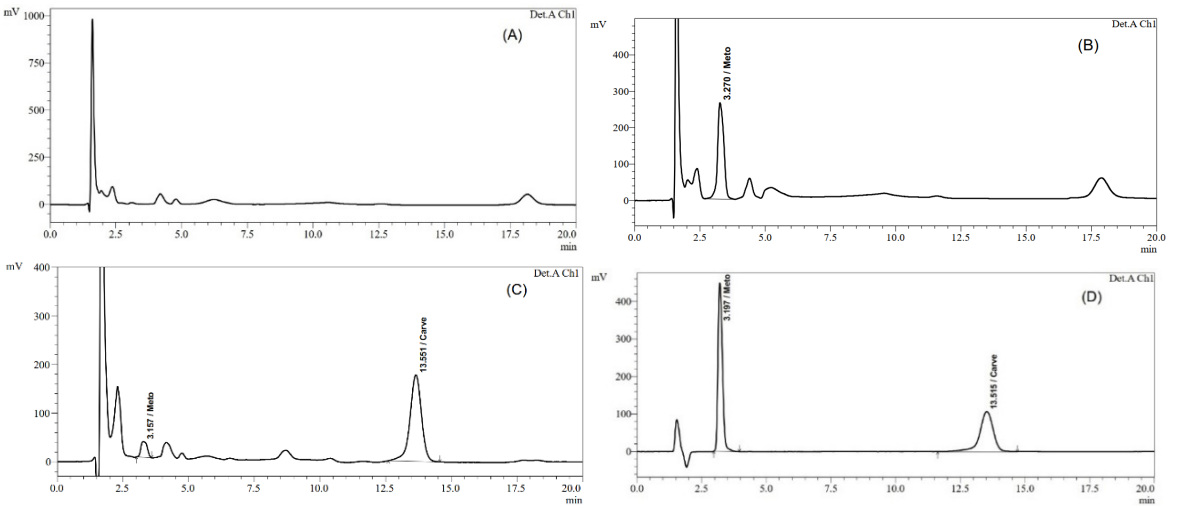

Figure 2. Chromatograms of (A) blank plasma; (B) plasma containing the IS; (C) plasma containing carvedilol and IS; (D) standard solutions of carvedilol and IS

There was a strong linear correlation between the concentration of carvedilol and the ratio of the peak areas of carvedilol and IS within the concentration range of 2.5-100 ng/mL, with the regression equation as y = 0.1104x + 0.0051. The linearity showed a good correlation with a good correlation coefficient of 0.9998, satisfying the requirement of $r^2 \geq 0.98$ (Table 2).

Table 2. The correlation between the ratio of the peak area and the concentration of carvedilol.

$C_{carvedilol}$ (ng/mL)	2.5	7.5	10	30	75	100
$S_{carvedilol}/S_{IS}$	0.2877	0.7989	1.1118	3.3133	8.3908	10.9718

$C_{carvedilol}$ is the concentration of carvedilol; $S_{carvedilol}/S_{IS}$ is the ratio of the peaks of carvedilol and IS.

The LLOQ was found to be 2.5 ng/mL for carvedilol and the carryover did not exceed 20% of the LLOQ. Besides, the accuracy and precision of the dilution with blank plasma were also within the acceptable range of 15% of the nominal concentrations and RSD, respectively.

The intra-day and inter-day precision and accuracy are presented in Table 3. All these values fell within the permissible limits of 85 - 115% of the test concentration and RSD ≤ 15%, respectively. Thus, the procedure met the requirements for accuracy and precision in quantifying carvedilol in biological fluids.

Table 3. Intra-day and Inter-day precision and accuracy of the assay

	Actual concentration (ng/mL)	**Concentration found (mean ± SD) ng/mL**	**Accuracy (mean ± SD) %**	**Precision (%RSD)**
Intraday (n = 6)	2.5	2.27 ± 0.13	90.94 ± 5.11	5.62
	7.5	7.56 ± 0.10	100.81 ± 1.34	1.33
	30	32.13 ± 1.07	107.10 ± 3.58	3.58
	75	80.74 ± 0.46	107.65 ± 0.62	0.57
Inter day (n = 6)	2.5	2.32 ± 0.15	92.63 ± 5.87	6.34
	7.5	7.30 ± 0.40	97.39 ± 5.18	5.31
	30	31.41 ± 1.27	104.70 ± 4.25	4.05
	75	79.06 ± 2.31	105.41 ± 3.08	2.92

For the recovery testing, it was determined at three concentrations of carvedilol and at concentration of 100 ng/mL of the IS. The average recovery of carvedilol and IS was found to be 94.41 ± 3.36% and 94.87 ± 2.82%, respectively (Table 4).

Table 4. Recovery of carvedilol from plasma

Carvedilol spiked concentration (ng/mL)	Recovery (mean ± SD)%	Precision (%RSD)
7.5	91.51 ± 4.58	5.01
30	92.50 ± 5.00	5.41
75	99.22 ± 0.50	0.50

Regarding the stability testing, the results are presented in Table 5. These values demonstrate that the sample exhibits good stability under all test conditions.

Table 5. Stability of carvedilol in plasma

	QC samples	Concentration found at 0h (mean ± SD) ng/mL	Concentration found at the last hour (mean ± SD) ng/mL	Deviation (mean ± SD) %	RSD (%)
Freeze-thaw	LQC	7.09 ± 0.40	7.46 ± 0.30	5.45 ± 5.48	4.08
	HQC	76.16 ± 3.69	74.74 ± 3.15	–1.65 ± 6.80	4.22
Short-term	LQC	7.09 ± 0.40	7.47 ± 0.57	5.40 ± 4.31	7.59
	HQC	76.16 ± 3.69	76.63 ± 3.05	0.92 ± 8.20	3.98
Long-term	LQC	7.09 ± 0.40	7.52 ± 0.39	6.17 ± 5.65	5.13
	HQC	76.16 ± 3.69	72.26 ± 2.58	- 4.99 ± 4.42	3.57

4. DISCUSSION

In this study, the chromatographic conditions were optimized, including the chromatographic column, mobile phase, and detection wavelength, to provide clear and symmetrical substance signals.

Two types of columns, InertSustain™ C18 (5 µm; 4.6x250 mm) and Zorbax Eclipse XDB-C8 (5 µm; 4.6x150 mm), were investigated. The results revealed no signal on the chromatogram when eluted on the C18 column, whereas a strong signal was observed with the C8 column. The C18 column, consisting of 18 carbon atoms bonded to the silica, is more densely packed than the C8 column, which has only 8 carbon atoms. This denser packing increases the surface area that the analyte molecules must traverse, prolonging interaction time in the elution and stationary phases. Consequently, the analyte elutes faster on the C8 column, leading to a shortened elution time and easy detection of the analyte signal on the chromatogram. This C8 column possesses column dimensions and packing particle size similar to the study of Gehr T W B et al. [15].

Several mobile phases and gradient programs were trialed using different proportions of water and organic solvents such as acetonitrile and methanol. Most studies employed phosphate buffer solutions and organic solvents as mobile phases. However, these buffer solutions are challenging to adjust and control pH over time, leading to random errors affecting validation results. In this study, a mobile phase consisting of 0.1% TFA in water and organic solvents was utilized, which proved optimal by improving the stability of the mobile phase while providing good resolution and symmetric peak shape.

In the previously published reports, the majority of detection wavelengths used fall within the maximum absorption range of carvedilol, resulting in a favorable signal for carvedilol on the chromatogram, while the signal for the IS was still suboptimal. The gradient program employed in this study includes wavelengths within the maximum absorption range of both carvedilol and the IS, which has been an optimization compared to previous studies, as both the signals for carvedilol and the IS were adequately recorded on the chromatogram with the retention time of 13.551 min and 3.157 min, respectively (Figure 2).

According to a previously published pharmacokinetic study of carvedilol by Gehr T W

B et al. [15], the concentration maximum (C_{max}) of carvedilol in plasma after administration of a 12.5 mg tablet was 53.4 ng/mL. Therefore, this LLOQ value meets the US-FDA and EMA requirements of being not less than one-twentieth of the C_{max} and is sufficiently sensitive to monitor the drug for various purposes, including pharmacokinetic studies.

The recovery of carvedilol in this study is higher compared to Rathod R. et al. [5] (69.90%) and approximately equivalent to Yilmaz B. et al. [6] (92.70% to 95.80%). However, the LLOQ in this method (2.5 ng/mL) is significantly improved compared to the LLOQ in the study by Yilmaz B. et al. (10 ng/mL).

5. CONCLUSION

From the study, a simple, accurate, and cost-effective method for quantifying carvedilol in human plasma has been developed and validated based on criteria outlined by the US-FDA and EMA guidelines. The specific chromatographic conditions include a Zorbax Eclipse XDB-C8 column (5 µm; 4.6 x 150 mm), a mobile phase consisting of 0.1% TFA in water, acetonitrile, and methanol (60:20:20; v/v/v), a flow rate of 1 mL/min and a gradient program with dual-wavelength detection. The method employed a straightforward sample preparation procedure and utilized a relatively small volume of plasma (1 mL), yet provided a relatively high sensitivity (LLOQ = 2.5 ng/mL). This makes it suitable for application in pharmacokinetic studies of carvedilol.

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