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HPLC-Method for the Quantification of Carvedilol in Human Plasma

BAPPADITYA CHATTERJEE, AYAN DAS and TAPAN K. PAL*

Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India

*Corresponding author: Fax: +91 33 24146186; Tel: +91 33 24146967; E-mail: proftkpal@gmail.com

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A simple and feasible reversed phase high performance liquid chromatographic (RP-HPLC) method coupled to UV detection for the determination and quantification of carvedilol in human plasma has been described. After sample preparation by one step liquid-liquid extraction with ethyl acetate, carvedilol and carbamazepine (internal standard) were chromatographically separated on a hypersil BDS, C_{18} (250 mm × 4.6 mm, 5 µm) column using a mobile phase composed of 10 mM potassium dihydrogen phosphate buffer (pH adjusted to 3.5) and acetonitrile in a ratio of 60:40 (v/v) at an isocratic flow rate of 1 mL/min. The wavelength of detection was set at 242 nm. Retention time of carvedilol and internal standard (carbamazepine) were 9.50 and 4.47 min, respectively. The assay was linear between the concentration ranges of 4-60 ng/mL for carvedilol in plasma with a satisfactory correlation coefficient (r^2) value above 0.9965. The extraction recovery of carvedilol in plasma at three quality control samples was ranged from 83.943-91.672 %. The method was specific and sensitive with the limit of quantification of 4 ng/mL. The accuracy and precision values obtained from six different sets of quality control samples analyzed in separate occasions were within the satisfactory limit. In stability study, carvedilol in human plasma was stable during storage and assay procedure. The developed and validated method is simple, sensitive and economical and can be successfully applied to the pharmacokinetic study of carvedilol in human volunteers.

Key Words: RP-HPLC, Carvedilol, Human Plasma.

INTRODUCTION

Carvedilol, 1-(9*H*-carbazol-4-yloxy)-3-[2-(2-methoxy-phenoxy)-ethylamino]-propan-2-ol is a non-selective β- and α_1 receptor blocking agent used as an antihypertensive drug[1,2]. It has multiple spectrums of actions like calcium antagonistic blocking activity[3] and inhibition of smooth muscle proliferation[4]. Carvedilol has been also used in the treatment of congestive heart failure and angina pectoris[5,6]. It has much greater antioxidant property than other β-blocker drugs[7].

Several bioanalytical methods have been published to quantify carvedilol in human plasma. Analysis of carvedilol in human plasma has been done by HPLC-MS/MS after chiral derivatization[8], hydrophilic interaction liquid chromatography with tandem mass spectroscopy[9], normal phase HPLC methods using chiral column[10], reversed phase HPLC method using fluorescence detection[11] or capillary electrophoresis with UV detection[12] have been developed to quantify carvedilol in human plasma. In this study we describe a rapid, selective and sensitive reversed phase high performance liquid chromatography method with UV detection to analyze and quantify carvedilol in human plasma. A very easy and one step liquid-liquid extraction method using commonly available solvent has been described in this paper. The method is validated as per the US FDA industry guidelines for bioanalytical method validation[13].

EXPERIMENTAL

Carvedilol and carbamazepine pure samples were obtained from Dr. Reddy's Lab Pvt. Ltd. (Hyderabad, India) and M/s Cosmas Pharmacals (Ludhiana, India) respectively as gift samples. Ethyl acetate of HPLC-grade and acetonitrile were purchased from Merck (Mumbai, India). HPLC-grade water (resistivity of 18 M cm) generated from Milli-Q water purification system was used throughout the analysis. The blank human plasma with EDTA-K_3 anticoagulant was collected from Clinical Pharmacological Unit (CPU) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

Instrumentation and chromatographic conditions: HPLC separation was performed on a Knauer high performance liquid chromatography system (Japan) consisting of a HPLC pump K-2501 model and a UV detector K-2501 model. Separation was achieved on a Thermo Hypersil BDS, C_{18} (250 mm × 4.6 mm, 5 µm) column using an isocratic flow of 1 mL/min at room temperature. Detection and quantification were done using chemitochrome software. The mobile phase was a mixture of 10 mM potassium dihydrogen phosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) and acetonitrile in a ratio of 60:40 (v/v). Samples were injected through the rheodyne injector system fitted with 50 µL fixed loop. The wavelength of detection of the analytes was 242 nm.

Preparation of stock and standard solution: Stock solutions of carvedilol and internal standard (carbamazepine) were prepared at concentration of 100 µg/mL in methanol. Stock solutions were stored at -20 ºC until they were used for working solutions by adding appropriate volume of mobile phase. Required concentration of working solutions were prepared by diluting the stock solution with the mobile phase afresh before the use.

Preparation of calibration standards and quality control samples: Plasma calibration standards of carvedilol (4, 10, 15, 20, 30, 45 and 60 ng/mL) were prepared by spiking appropriate aliquots of working solution of carvedilol in blank plasma from healthy, non-smoking volunteers. Final concentration of internal standard in plasma was 100 ng/mL. Four levels of quality control samples at a concentration of 4 (lower limit of quantification, *i.e.*, LLOQ), 12 (low-), 35 (medium-) and 50 (high-) ng/mL were prepared from a pool of six different sources of blank plasma using the stock solution of carvedilol.

Sample preparation: For calibration standards, an aliquot quantity of 0.9 mL plasma sample was mixed with 0.1 mL of internal standard in a 10 mL stopper test tube. To it 4 mL of organic solvent (ethyl acetate) was added and after another mixing step for 15 min the solution was centrifuged at 5000 rpm for 20 min. The organic layer was separated and evaporated to dryness at 35 ºC under N_2 atmosphere. The residue was reconstituted in 200 µL of mobile phase by vortex mixing and filtered through 0.22 µm membrane filter. Finally 50 µL of the prepared sample was injected into the HPLC system through fitted loop.

Validation of method: The developed method was validated for specificity, linearity, accuracy, precision, recovery and stability to meet the acceptance criteria of food and drug administration (FDA) industry guidance for bioanalytical method validation[13].

Specificity: The specificity of the method was investigated by analyzing blank human plasma extract for endogenous matrix constituents and an extract spiked with carvedilol along with internal standard.

Linearity: The linearity was determined by plotting the peak-area ratio(y) of carvedilol to internal standard *versus* the nominal concentration(x) of carvedilol in plasma. The calibration curve was obtained by the least-squares linear regression (no weighing factor) presented with their correlation coefficients over the concentration range of 4-60 ng/mL for carvedilol and 100 ng/mL for internal standard in plasma samples.

Accuracy and precision: Six replicates of three quality control samples (low-, medium- and high- concentration) were used to determine the accuracy and precision of the method. Accuracy was determined by calculating the percentage deviation observed in the analysis of quality control samples and expressed as relative error (RE). Intra day and inter day precision assessment was done to determine the precision of the method and the results are expressed as the percentage coefficient of variance (CV %).

Extraction recovery: The extraction recovery of analyte at three quality control samples was determined by comparing peak areas from plasma samples spiked with particular standard working solution of analyte before extraction with the corresponding standard solutions without extraction. The recovery of IS at concentration of 100 ng/mL was determined in the same way. Six replicates of each quality control samples were taken to study the extraction recovery.

Stability: The stability of carvedilol in plasma was evaluated with four studies; short-term, long-term and freeze-thaw stability study as well as stability in ambient temperature. Six replicates of three quality control samples at concentration of 12 ng/mL (LQC), 35 ng/mL (MQC) and 50 ng/mL (HQC) was prepared and then subsequent HPLC analysis was carried out as described previously.

The quality control samples were kept at room temperature for 24 h, extracted and then analyzed for short-term stability study. The long-term stability study was carried out with plasma samples spiked with carvedilol, which were stored -20 ºC for 1 and 3 months, then extracted and analyzed.

The freeze-thaw stability study was evaluated by comparing the quality control samples that had been frozen and thawed three times, with the plasma samples thawed once.

RESULTS AND DISCUSSION

Specificity: Carvedilol and internal standard exhibited retention times at 9.50 and 4.57 min, respectively under the described chromatographic conditions. There is no significant interference from endogenous substances in drug-free plasma at the retention time of the analytes was observed (Fig. 1).

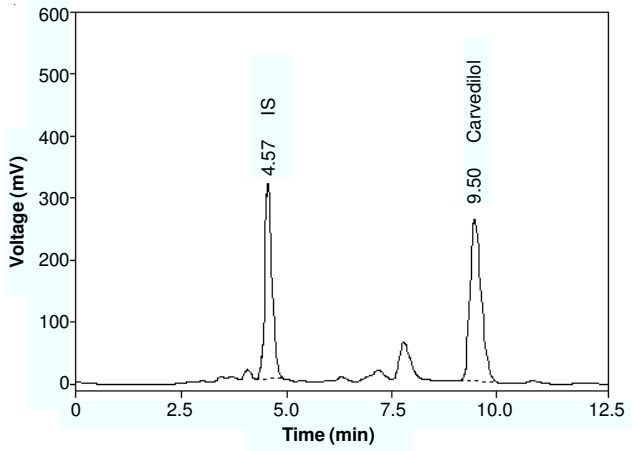

Fig. 1. Chromatogram of blank plasma spiked with drug and internal standard (carbamazepine)

Linearity: Excellent linearity was noted between the peak-area ratios of carvedilol to internal standard *versus* concentration of carvedilol. The regression coefficient value of the calibration curve was 0.9965. The representative regression equation for the calibration curve was y = 0.0208x - 0.0343 over the concentration range of 4-60 ng/mL for carvedilol in human plasma.

Accuracy and precision: As described in the Table-1, for each quality control level of carvedilol, the intra day and inter day precision values of carvedilol was ranged from 1.528-2.469 and 2.046-3.797 %, respectively. At the same concentrations, the values for accuracy expressed as percentage relative error shown in Table-1 was within 0.5 % indicating the acceptable accuracy of the HPLC method to determine carvedilol in human plasma.

TABLE-1
ASSESSMENT OF ACCURACY AND PRECISION FROM QUALITY-CONTROL SAMPLE

QC Sample (ng/mL)	Mean conc. found (ng/mL)		SD		CV (%)		RE (%)	
	Intra day (n = 12)	Inter day (n = 24)	Intra day (n = 12)	Inter day (n = 24)	Intra day (n = 12)	Inter day (n = 24)	Intra day (n = 12)	Inter day (n = 24)
12	12.041	11.813	0.202	0.449	1.679	3.797	0.344	-1.560
35	33.983	34.262	0.839	0.700	2.469	2.046	-2.906	-2.107
50	49.575	49.432	0.757	1.064	1.528	2.153	-0.849	-1.135

SD = Standard deviation; CV (%) = Coefficient of variation [(SD/mean) × 100]; RE (%) = Relative error [{(conc. found – conc. added)/conc. added} × 100]; n = number of replicates.

Extraction recovery: As described in Table-2, the recoveries (mean) of carvedilol at 12 (low-), 35 (medium-) and 50 (high-) ng/mL quality control samples were within a range of 83.943-91.672 % respectively. The recovery (mean) of internal standard was 84.839 % at the concentration used in the assay.

TABLE-2
ANALYSIS OF EXTRACTION RECOVERY OF ANALYTES FROM QC SAMPLES (n = 6)

Analyte	QC Sample (ng/mL)	Extraction recovery (%)	CV (%)
Carvedilol	12	83.943	1.397
	35	87.876	2.311
	50	91.672	1.970
Carbamazepine	100	84.839	1.830

CV (%) = Coefficient of variation [(SD/mean) × 100]; n = number of replicates.

Stability: As per the procedure described in the experimental part, the stability study of carvedilol in plasma was carried out with six replicates of three levels of quality control samples. Results of stability study are described in Table-3.

TABLE-3
STABILITY STUDY OF ANALYTES AT DIFFERENT CONDITIONS (n = 6)

Storage condition	Low-QC (12 ng/mL)	Medium-QC (35 ng/mL)	High-QC (50 ng/mL)
3 Freeze/thaw cycle	93.148 (1.146)	95.524 (1.944)	96.438 (2.128)
24 h ambient	94.366 (2.092)	96.401 (2.349)	97.786 (2.385)
1 month frozen (-20 ºC)	94.936 (2.095)	95.937 (1.999)	98.108 (2.112)
3 month frozen (-20 ºC)	95.065 (1.202)	96.605 (2.380)	98.112 (1.375)

Data presented in this table are the percentage of measured value *versus* theoretical value with CV % in parenthesis; n = number of replicates.

The calculated values of coefficient of variance (CV %) and accuracy for quality control samples undergoing three freeze-thaw cycles are ≤ 2.128 and ≥ 93.148 %, respectively. quality control samples storing at ambient for 24 h exhibited CV % value as ≤ 2.385 and an accuracy of ≥ 94.366 %.

Long term frozen storage stability was tested at 1 and 3 month after quality control sample pools were prepared and stored at -20 ºC. The 1 month stability data of all three quality control samples showed an accuracy of more than 94.936 % with CV % value less than 2.112 and the 3-month stability data had an accuracy of more than 95.065 % with CV % value less than 2.380 in plasma.

Conclusion

The described RP-HPLC method involves a rapid, specific, sensitive and stable assay for the determination of carvedilol in plasma. Sample preparation under the proposed method involves a quick and simple liquid-liquid extraction with easily available solvent in comparison to the methods reported till date. The chromatographic run time of the method is low as well as the mobile phase composition is also very simple. Therefore the method permits the analysis of a large number of samples at a comparatively low cost and in less time. The method can be successfully applied to the pharmacokinetic study of carvedilol on human volunteers after the administration of it's dosage form.

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