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Short communication

Quantification of carvedilol in human plasma by liquid chromatography using fluorescence detection: Application in pharmacokinetic studies

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Abstract

A simple, rapid and sensitive isocratic reversed-phase HPLC method with fluorescence detection using a monolithic column has been developed and validated for the determination of carvedilol in human plasma. The separation was performed on a Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column with an isocratic mobile phase consisting of 0.01 M disodium hydrogen phosphate buffer–acetonitrile (40:60, v/v) adjusted to pH 3.5. The sample preparation involves protein precipitation procedure and analytical recovery was complete. Letrozole was used as internal standard. The assay enables the measurement of carvedilol for therapeutic drug monitoring with a minimum quantification limit (LOQ) of 1 ng ml^{-1}. The excitation and emission wavelengths were set at 240 and 340 nm, respectively. The calibration curve was linear over the concentration range 1–80 ng ml^{-1}. The coefficients of variation for inter-day and intra-day assay were found to be less than 8.0%.
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Keywords: Carvedilol; Plasma; HPLC; Monolithic column

1. Introduction

Carvedilol is a non-cardioselective beta blocker which is used in the management of hypertension and angina pectoris. It is also used to reduce mortality in patients with left ventricular dysfunction following myocardial infarction [1]. Carvedilol is well absorbed from the gastro-intestinal tract with peak concentrations in plasma occurring about 1–2 h and it is highly protein bound (>98%) [2]. Several chromatographic methods have been developed for the determination of carvedilol in biological fluids [3–10]. Most of these methods are focused on the separation of enantiomers [6–8] or deal with analyses in urine and various biological materials [9,10], which is usually not necessary for bioequivalence studies. Only few methods have been reported for determination of carvedilol in human plasma or serum [3–5]. However, these methods have various limitations, including time-consuming sample clean-up and laborious extraction steps, low sensitivity and long run times which are not suitable in all conditions. Two sensitive LC–MS/MS assays have been also reported for the stereoselective analyses of carvedilol in plasma [11,12]. Both the methods are very sensitive, having low quantitation limits. However, these methods are not suitable for bioequivalence studies and other routine pharmacokinetic purposes due to using of complex and time-consuming derivatization procedure with chiral reagents. Moreover, this technique is not available for most laboratories because of its specialty requirement and financial reasons. This paper describes the development and validation of a simple, rapid and sensitive isocratic reversed-phase HPLC method for the determination of carvedilol in human plasma using a monolithic column with fluorescence detection. Recently, monolithic columns have generated interest as alternative to particulate columns in liquid chromatography due to their simple preparation procedure, unique properties and excellent performance, especially for separation of drugs in biological samples [13,14]. Their properties enhance the speed of the separation process and reduce backpressure and unspecific binding without sacrificing resolution [15]. In our method, separation was performed on a reversed-phase monolithic column, which has lower separation impedance comparing to the particulate packings, and therefore it allows easy

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optimizing chromatographic conditions to obtain desirable resolution in a short time. The sample preparation only involves protein precipitation and no evaporation step is required. Also, the use of a small sample volume and short analysis time provides advantages for analysis of carvedilol in plasma. We also demonstrate the applicability of this method for pharmacokinetic studies in humans.

2. Experimental

2.1. Chemicals

Carvedilol and letrozole were supplied by Darupakhsh Pharmaceuticals (Tehran, Iran). Carvedilol is available as oral tablet containing 25 mg of carvedilol and other inactive ingredients. HPLC-grade acetonitrile and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation and purified additionally with a Milli-Q system.

2.2. Instruments and chromatographic conditions

The chromatographic apparatus consisted of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2600 fluorescence detector connected to a model Eurochrom 2000 integrator, all from Knauer (Berlin, Germany). The separation was performed on Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column from Merck (Darmstadt, Germany). The excitation and emission wavelengths were set at 240 and 340 nm, respectively.

The mobile phase was a mixture of 0.01 M disodium hydrogen phosphate buffer–acetonitrile (60:40, v/v) adjusted to pH 3.5 at a flow rate of 2 ml min^{-1}. The mobile phase was prepared daily and degassed by ultrasonication before use. The mobile phase was not allowed to recirculate during the analysis.

2.3. Standard solutions

Stock solutions (8 mg ml^{-1}) of carvedilol and letrozole (10 mg ml^{-1}) were prepared in methanol. Then 10, 50, 100, 200, 400 and 800 ng ml^{-1} working standards of carvedilol were prepared in plasma from the stock solution and stored at +4 °C.

2.4. Sample preparation

To 450 μl of plasma in a glass-stoppered 15 ml centrifuge tube were added 50 μl of letrozole as internal standard (10 μg ml^{-1}) and 500 μl of acetonitrile. After mixing (30 s), the mixture centrifuged for 10 min at 8000 × *g*. Then 20 μl of supernatant was injected into liquid chromatograph.

2.5. Stability

The stability of carvedilol was assessed for spiked plasma samples stored at −20 °C for 1 month and at ambient temperature for at least 12 h. The stability of stock solutions stored at −20 °C was determined for up to 1 month by injecting appropriate dilutions of stocks in methanol on day 1, 15 and 30 and comparing their peak areas with fresh stock prepared on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

2.6. Plasma standard curve

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at −20 °C. After thawing, stock solution of carvedilol was added to yield final concentrations of 1, 5, 10, 20, 40 and 80 ng ml^{-1}. Internal standard solution was added to each of these samples to yield a final concentration of 1000 ng ml^{-1}. The samples were then prepared for analysis as described above.

2.7. Selectivity and specificity

Control human plasma, obtained from 12 healthy volunteers, was assessed by the procedure as described above and compared with respective plasma samples to evaluate selectivity of the method. Sotalol, propranolol and atenolol were also tested for potential interferences.

2.8. Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of carvedilol to pool plasma (quality control samples). For intra-day precision and accuracy five replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days.

2.9. Limit of quantification (LOQ) and recovery

For the concentration to be accepted as LOQ, the percent deviation from the nominal concentration (accuracy) and the relative standard deviation must be ±20% and less than 20%, respectively, considering at least five times the response compared to the blank response. The relative analytical recovery for plasma at three different concentrations of carvedilol (5, 30 and 60 ng ml^{-1}) was determined. Known amounts of carvedilol were added to drug-free plasma and the internal standard was then added. The relative recovery of carvedilol was calculated by comparing the peak areas for extracted carvedilol from spiked plasma and a standard solution of carvedilol in acetonitrile containing internal standard with the same initial concentration (six samples for each concentration level).

2.10. Biological samples

Twelve male healthy volunteers were included in this study. The study protocol was approved by the Ethics Committee of Shaheed Beheshti University of Medical Sciences and written informed consent was obtained from the volunteers. Carvedilol was administered in a single dose of 25 mg to the volunteers after over night fasting. Plasma samples were collected at 0.5,

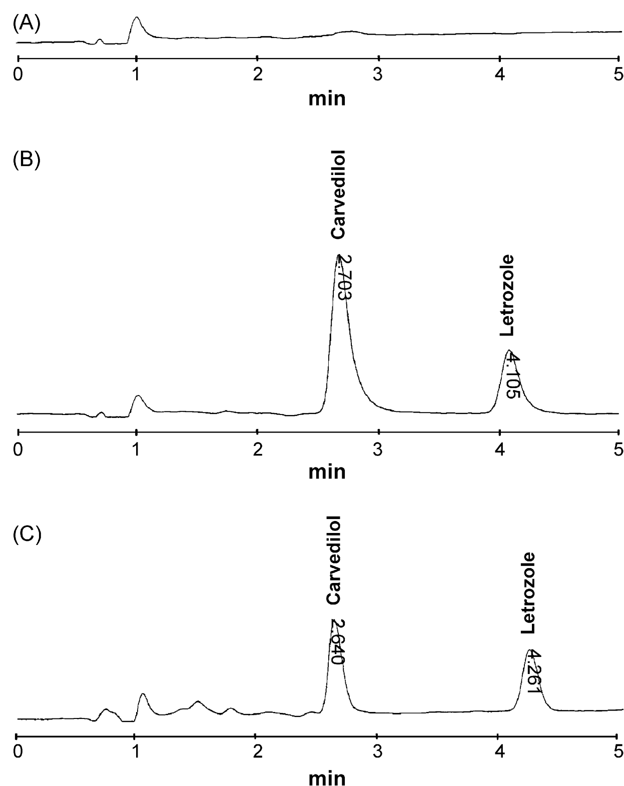

Fig. 1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 60 ng ml^{-1} carvedilol and 1000 ng ml^{-1} letrozole (internal standard); (C) plasma sample from a healthy volunteer 2 h after oral administration 25 mg of carvedilol.

1, 1.5, 2, 2.5, 3, 4, 5, 7, 10 and 24 h after dosing and then frozen immediately at −20 °C until assayed.

3. Results and discussion

Under the chromatographic conditions described, carvedilol and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Fig. 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of carvedilol and letrozole were 2.7 and 4.1 min, respectively. None of the drugs mentioned above interfered with analytes peaks as well. The calibration curve for the determination of carvedilol in plasma was linear over the range 1–80 ng ml^{-1}. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (r) for calibration curves were equal to or better than 0.999. The relative standard deviation (R.S.D.) values of the slope were less than 4%. For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves (Table 1). The relative analytical recovery for plasma at three different concentrations of carvedilol (5–60 ng ml^{-1}) was determined. As shown in Table 2. The average recovery was 98.1 ± 2.2% (n = 6). Accordingly, the recovery of letrozole at concentration of 1000 ng ml^{-1} was 100.4 ± 1.6 (n = 5). The limit of quantification (LOQ), as previously defined, was 1 ng ml^{-1} for carvedilol. This is sensitive enough for drug monitoring and other purposes such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of carvedilol. As given in Table 3, coefficients of variation were less than 8%, which is acceptable for the routine measurement of carvedilol. Stability was determined for spiked plasma samples under the conditions as previously described. The results showed that the samples were stable during the mentioned conditions. The aim of our study was to develop a rapid and sensitive method for analysis of cavedilol in biological samples for pharmacokinetic purposes and related studies. This method is well suited for routine application in the clinical laboratory because of the speed of analysis and simple extraction procedure. Owing to use of the monolithic column, which has lower separation impedance comparing to the particulate packings, much faster separations are possible the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. Accordingly, the chromatographic elution step is undertaken in a short time (less than 5 min) with high resolution. The sample preparation only involves protein precipitation and no evaporation step is required. Over 600 plasma samples were analyzed by this method without any significant loss of resolution. No change in the column efficiency and backpressure was also observed

Table 2
Relative recovery of carvedilol from plasma

Carvedilol spiked concentration (ng ml^{-1})	Carvedilol concentration found (n = 6)	Recovery (mean ± S.D.)%
5	4.8	96.0 ± 1.9
30	29.8	99.3 ± 2.1
60	59.3	98.9 ± 2.7

Table 3
Reproducibility of the analysis of carvedilol in human plasma (n = 5)

Concentration added (ng ml^{-1})	Concentration measured (mean ± S.D.)	
	Intra-day	Inter-day
5	4.92 ± 0.35 (7.11)	5.03 ± 0.29 (5.76)
30	30.20 ± 1.70 (5.63)	29.70 ± 1.67 (5.62)
60	59.49 ± 1.93 (3.24)	60.22 ± 1.88 (3.12)

Values in parentheses are coefficients of variation (%).

Table 1
Assay linearity

	Coefficient of the linear regression analysis (r ± S.D.)	Slope ± S.D.	Intercept ± S.D.
Intra-assay (n = 6)	0.9995 ± 6.20 × 10^{-4} (R.S.D. = 0.0620%)	0.0381 ± 0.0010 (R.S.D. = 2.62%)	0.0065 ± 0.0004
Inter-assay (n = 9)	0.9992 ± 5.20 × 10^{-4} (R.S.D. = 0.0520%)	0.0384 ± 0.0012 (R.S.D. = 3.13%)	0.0067 ± 0.0005

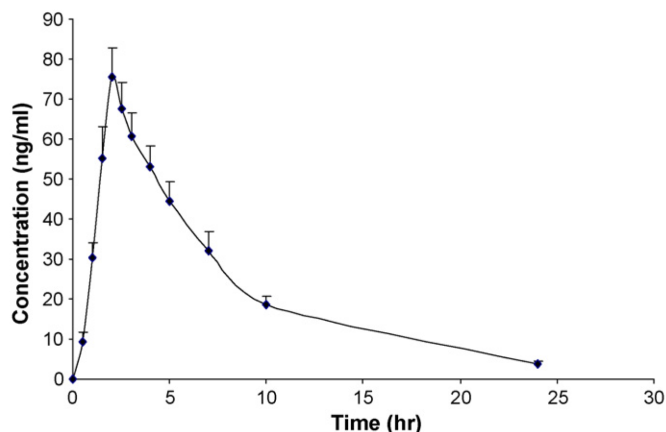

Fig. 2. Mean plasma concentrations (+S.D.) of carvedilol in healthy volunteers ($n = 12$) after 25 mg single oral dose.

Table 4
Pharmacokinetic parameters of carvedilol in healthy volunteers following a single oral dose of 25 mg of carvedilol

Parameter	Result (mean ± S.D.)
T_{max} (h)	1.95 ± 0.25
C_{max} (ng ml^{-1})	75.35 ± 4.83
AUC_{0-t} (ng h ml^{-1})	560.93 ± 44.33
K_{el} (h^{-1})	0.121 ± 0.004
$T_{1/2}$ (h)	5.72 ± 0.23

over the entire study time, thus proving its suitability. In this study plasma concentrations were determined in 12 healthy volunteers, who received 25 mg of carvedilol each. Fig. 2 shows the mean plasma concentration–time curve of carvedilol: plasma concentration reached a maximum 1.95 ± 0.25 h after dosing with a level of 75.35 ± 4.83 ng ml^{-1}. The derived pharmacokinetic parameters of 12 healthy volunteers are summarized in Table 4. These pharmacokinetic parameters are in good agreement with that found previously [16].

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