A new HPLC Method for Determination of Carvedilol in Human Plasma and its Application in Bioequivalence Studies

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A simple, rapid and sensitive isocratic reversed-phase high performance liquid chromatography method for the determination of carvedilol in human plasma has been developed. After extraction with methanol chromatographic analysis of carvedilol in plasma was achieved on a μ-bondapack C_{18} column using acetonitrile- potassium dihydrogen phosphate (30: 70v/v) mixture, pH 2, as mobile phase. The flow rate was set at 1 ml/min and the emission and excitation were 350 and 238 respectively. The lower limit of detection was 0.05 ng/ml and lower limit of quantization was 0.2ng/ml. The intra and inter-day precisions (CV %) of the quality control samples were 2.45- 7.27 and 0.52-7.92% respectively. The recovery of method was %99.48±8.5. The method was applied to a bioequivalence study in human.

Keywords: Carvedilol; Human plasma; HPLC; Bioequivalence

The b-blocker carvedilol is effective against hypertension and can prolong the survival of patients with chronic heart failure. Carvedilol is administered as a racemic drug. Carvedilol is a potent antihypertensive agent with a dual mechanism of action. At relatively low concentrations it is a competitive ²-adrenoceptor antagonist and a vasodilator, whereas at higher concentrations it is also a calcium channel antagonist. The antihypertensive activity of carvedilol is characterized by a decrease in peripheral vascular resistance, resulting from the vasodilator activity of the compound, with no reflex tachycardia, as a result of ²-adrenoceptor blockade. The antihypertensive activity of carvedilol is associated with an apparent "renal sparing" effect in that the reduction in mean arterial blood pressure does not compromise renal blood flow or urinary sodium excretion. Studies on the mechanism of action of carvedilol indicate that the compound is a potent competitive antagonist of $^2_1$- and $^2_2$-adrenoceptors with a dissociation constant (K_B) of 0.9 nM at both ²-adrenoceptor subtypes. Carvedilol is also a potent $\pm_1$-adrenoceptor antagonist ($K_B = 11$ nM), which accounts for most, if not all, of the vasodilating response produced by the compound. At concentrations above 1 ¼M, carvedilol is a calcium channel antagonist. This activity can be demonstrated in vivo at doses that represent the higher end of the anti-hypertensive dose-response curve. Although the calcium-channel blocking activity of carvedilol may not contribute to the antihypertensive activity of the compound, it may play a prominent role in certain peripheral vascular beds, such as the cutaneous

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circulation, where marked increases in blood flow are observed. The data indicate that carvedilol is an antihypertensive agent that is both a β-adrenoceptor antagonist and a vasodilator. The vasodilating activity of carvedilol results largely from α_1-adrenoceptor blockade, and its β-adrenoceptor blocking activity prevents reflex tachycardia. In some regional vascular beds, such as the cutaneous circulation, the calcium-channel blocking activity of carvedilol may be responsible for increasing the blood flow.[1] carvedilol is rapidly and extensively absorbed following oral administration, with absolute bioavailability of approximately 25% to 35% due to a significant degree of first-pass metabolism. Following oral administration, the apparent mean terminal elimination half-life of carvedilol generally ranges from 7 to 10 hours. Plasma concentrations achieved are proportional to the oral dose administered. When administered with food, the rate of absorption is slowed, as evidenced by a delay in the time to reach peak plasma levels, with no significant difference in extent of bioavailability. Carvedilol is extensively metabolized. Following oral administration of radiolabelled carvedilol to healthy volunteers, carvedilol accounted for only about 7% of the total radioactivity in plasma as measured by area under the curve (AUC). Less than 2% of the dose was excreted unchanged in the urine. Carvedilol is metabolized primarily by aromatic ring oxidation and glucuronidation. The oxidative metabolites are further metabolized by conjugation via glucuronidation and sulfation. The metabolites of carvedilol are excreted primarily via the bile into the feces. Demethylation and hydroxylation at the phenol ring produce 3 active metabolites with β-receptor blocking activity. Based on preclinical studies, the 4'-hydroxyphenyl metabolite is approximately 13 times more potent than carvedilol for β-blockade.[2,3] Several spectrophotometric , HPLC, HPTLC, atomic absorption, LC/MS/MS, GC-MS methods have been reported for the analysis of carvedilol in pharmaceutical dosage forms, [4-9] and in human plasma [10-14]. The aim of this study was to develop a simple, rapid, sensitive and reliable HPLC method with fluorescence detection for quantization of carvedilol in human plasma samples after protein precipitation with acetonitrile. The method was validated according to procedures and acceptance criteria based on FDA guideline and recommendations of ICH, to provide enough selectivity, sensitivity and reliability in pharmacokinetic and bioequivalence studies.

MATERIALS AND METHODS

Acetonitrile (HPLC grade), potassium dihydrogen phosphate, phosphoric acid (analytical grade), were purchased from Merck. Carvedilol and letrezol were USP reference standard.

Sample and standard solutions preparation:

To a 0.5 ml aliquot of plasma, 30 µl letrezol (50 µg/ml) as Internal standard, 1ml alkalin methanol (pH=8) were added and vortexed for 30 s. The mixture was centrifuged at 3000 rpm for 20 min. The upper organic phase was transferred to another tube and evaporated under air. 500 µl mobile phase was added to dry residue and sample was dissolved and centrifuged at 4000 rpm for 5 min and 20 µl of the supernatant was injected to the chromatographic system.

Instrument and chromatographic conditions:

Analyses were performed on a Knauer model K-1001 pump equipped with a RF-10AXL fluorescent detector. Chromatography was performed at room temperature on a µ-bondapack C_{18} column (5 µm particle size, 25 cm × 4.6 mm I.D.). The mobile phase consisted of acetonitrile - potassium dihydrogen phosphate (30:70) mixture, pH 2 which was adjusted using phosphoric acid. The flow rate was set at 1 ml/min and the emission and excitation were 350 and 238 nm respectively.

Method validation

Validation was accomplished through determination of specificity, recovery, linearity, quantization limit, precision and accuracy.[15] Specificity was investigated by analyzing six drug-free plasma samples for interference of endogenous compounds. For calibration curve different concentrations of carvedilol 0.2-10 ng/ml and 20-100 ng/ml in plasma were prepared by adding required volume of working solutions to blank plasma. Plasma calibration curve was prepared by taking area ratio of analyte to internal standard as Y-axis and concentration of analyte (ng/ml) as X-axis. Linearity of the standard curve was evaluated using least-squares linear regression analysis. The intra and inter-day precisions (CV %) of the assay procedure was determined by trice analysis of

quality control plasma samples (5, 20 and 80 ng/ml) at the same day and three different days. Recovery was determined by comparing the response of three pre-treated quality control plasma samples in three levels (5, 20, 80 ng/ml) with the absolute peak area of un-extracted samples containing the same concentration of the drug as 100%.

Application

The validated method was used in bioequivalence study of carvedilol. It was an open, randomized crossover study to asses relative bioavailability of carvedilol in twelve healthy volunteers following single dose administration of carvedilol as 25 mg tablet. Test preparation was carvedilol 25 mg tablet manufactured by an Iranian pharmaceutics Co. The tablet containing 25 mg of carvedilol, manufactured by Hexal was used as reference preparation. The blood collecting times were 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6.5, 8 10 and 24 h after oral administration of 25 mg carvedilol reference and test. The plasma samples were analyzed by the described method. The pharmacokinetic parameters like area under the plasma-concentration-time curve from time zero to the last measurable carvedilol sample time and to infinity (AUC_{0-t} and AUC_{0-inf}), maximum concentration (C_{max}), time to maximum concentration (T_{max}) were determined for the period of 0 to 24 h.

Statistical analysis

The analysis of variance was performed on data for differences between and within the subspecies using the ANOVA (SPSS ver. 10). Mean
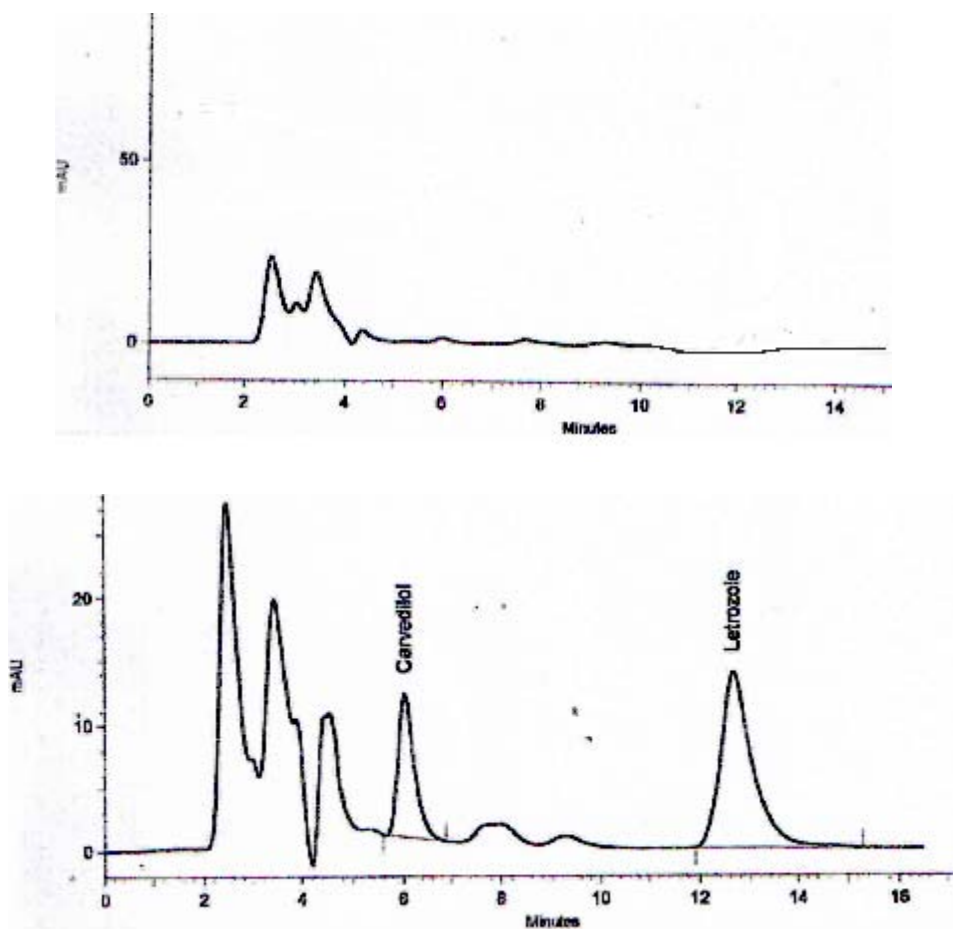

Fig.1. Chromatograms of (A) Blank human plasma; (B) Human plasma after administration of carvedilol tablet (rt=6min) and letrezol (rt=12.4 min)

separations were determined by least significant difference (LSD) at P d"0.05%.

RESULTS AND DISCUSSION

Some HPLC, HPTLC and atomic absorption methods have been developed for determination of carvedilol in pharmaceutical dosage form, but few methods have been developed for quantization of carvedilol in plasma. One of the challenging aspects of method development in quantitative analysis is the complexity of the analysis method. The simpler the method the better it could be conducted by different operators and in different labs. However other parameters of a quantitative method such as accuracy and precision demand more complex processes. The proposed method is suitable for carvedilol quantification in plasma samples. It showed specificity, since no interfering peaks from endogenous components of plasma were observed. Representative chromatogram of blank plasma and spiked plasma with carvedilol and internal standard are shown in figure 1. Retention time for the carvedilol and internal standard were 6 min and 12.4 min, respectively. Three dimensional and contour plot views of the chromatograms also
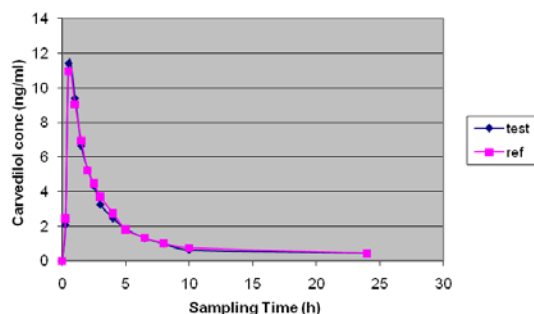
Fig. 2. Mean plasma concentration-time curve for test and reference preparation following single oral administration of carvedilol 25 mg tablet in 24 healthy volunteers

confirmed the complete separation. The chromatographic run time was 17 minutes for plasma sample analysis. The method was linear over the range of 0.2 to 10 ng/ml and 20 to 100ng/ml the calibration curve could be described by the equation y=0.04x+0.007 (r^2=0.99) and y=0.03x+0.16 (r^2=0.99) respectively.

The limit of detection (LOD) and quantization (LOQ) were 0.05, 0.2 ng/ml respectively. The intra and inter-day precisions (CV %) of the quality control samples were 2.45- 7.27 and 0.52-7.92% respectively. The accuracy of this bioanalytical method was %99.48±8.5. (Table1). It can be concluded that the accuracy and precision of carvedilol satisfied the acceptance criteria, and the proposed analytical method gave reproducible intra- and inter-day precision. The above mentioned method was used in the plasma analysis of a bioequivalence study of carvedilol as described earlier. The mean plasma level of carvedilol for test and reference preparation after the oral administration of a 25 mg single dose of carvedilol in 24 health human volunteers are given in Fig 2. Maximum plasma concentration (C_{max}) ranged from 21.49 to 33.91 ng/ml at 30 min (T_{max}). Also the mean value of area under the concentration time curve (AUC_{0-24}) obtained was 37.72 ng h/ml and 36.12 ngh/ml for test and reference respectively. Statistical comparison of the AUC_{0-24}, C_{max} and T_{max} clearly indicated no significant difference between test and reference, 25 mg tablets, in any of the calculated pharmacokinetic parameters and these values are entirely within the bioequivalence acceptance range of 80-125%. Therefore, this analytical method is applicable to pharmacokinetic studies.

The main advantage of this method is the use of precipitation for purification, which is easily and fast in comparison with other purification and extraction methods. This HPLC method is reliable, reproducible and sensitive with respect to validation parameters. It can be used as an assay

Table 1. Precision and Recovery of Carvedilol Assay in Human Plasma (n=3)

Spiked (ng/ml)	Intra-day			Recovery%	Inter-day		
	Found	SD	CV (%)		Found	SD	CV (%)
5	5.46	0.13	2.45	109.2	5.49	0.03	0.52
20	19.18	0.59	3.1	95.9	18.91	0.74	3.91
80	74.68	5.43	7.27	93.35	75.64	2.31	3.06

method in the study of carvedilol pharmacokinetics as well as bioavailability/ bioequivalence studies.

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