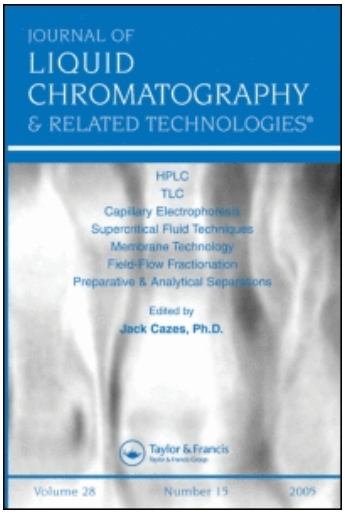

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New RP-HPLC Method with UV-Detection for the Determination of Carvedilol in Human Serum

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Abstract: Carvedilol has been used as an antihypertensive drug and also possesses antioxidant and antiproliferative effects. A simple and sensitive analytical method for carvedilol in human serum by using high performance liquid chromatography (HPLC) was developed. The method employs a liquid-liquid extraction for isolation and sample concentration followed by reversed phase liquid chromatography (RPLC) analysis using ultraviolet (UV) detection at 238 nm. Analytes were extracted from serum samples, that were previously mixed with 300 μL of 0.1 N sodium hydroxide solution into an n-hexane, dichloromethane (7:3) solvent system. The mobile phase was made of acetonitrile, 15 mM orthophosphoric acid (37:63), and 0.25% v/v of triethylamine, with a flow rate of 1 mL/min. Serum samples containing the carvedilol and internal standard, amitriptyline were eluted through a C8, Kromasil KR 100 5C8 column. Retention times of carvedilol and amitriptyline were 6.10 min and 8.44 min, respectively. The intra day and inter day coefficient of variation and percent error values of the assay method were less than 5%. The calibration curve was linear over a concentration range of 5–500 ng/mL. The extraction recovery of carvedilol is more than 75%. The validated method was applied to a pharmacokinetic study of carvedilol in human serum following the administration of a single carvedilol tablet (6.25 mg). Such a method would be ideally suitable for estimation of the drug for pharmacokinetic studies in human volunteers after oral administration of a single or multiple dosage(s) of carvedilol.

Keywords: Carvedilol, HPLC, UV detection, Human serum, Pharmacokinetics

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INTRODUCTION

Carvedilol (±)−1-(Carbazol-4-yloxy)-3-{[2-(o-methoxyphenoxy)ethyl]amino}-2-propanol (Figure 1), is a β receptor blocker and antihypertensive drug with a multiple action spectrum,[1] and it also has vasodilating properties that are attributed mainly to its blocking activity at α1 receptors. Carvedilol is used in the treatment of mild to moderate hypertension and angina pectoris.[2] Carvedilol undergoes extensive first pass liver metabolism that results in an absolute bioavailability of about 25%.[3]

Several techniques have been reported for carvedilol quantification in biological samples, plasma,[4] serum,[5] urine,[6,7] and cardiac tissue.[8] These published techniques include complicated sample preparation, column switching procedure,[9] fluorescence measurement,[4,10] and mass spectrometry.[7]

The main objective of this study was to develop a simple, sensitive, reliable, time, and money saving fully validated HPLC method with UV detection, for the determination of carvedilol in human serum. We also demonstrated the applicability of this method for pharmacokinetic studies in humans.

EXPERIMENTAL

Materials

Carvedilol and amitriptyline hydrochloride pure samples were gifted by Sun Pharmaceuticals, Baroda, India and Torrent Pharmaceuticals, India, respectively. Acetonitrile, methanol (HPLC grade) were obtained from Rankem, India. Triethyl amine (AR grade) was obtained from SD fine chemicals, orthophosphoric acid (GR grade), dichloromethane (GR grade), and n-hexane (GR grade) were obtained from E-Merck, India. Double distilled water was used during the entire HPLC procedure.

Standard Solutions

Primary stock solutions of 1 mg/mL of carvedilol and amitriptyline were prepared in methanol and stored at 4°C. Appropriate dilutions of carvedilol

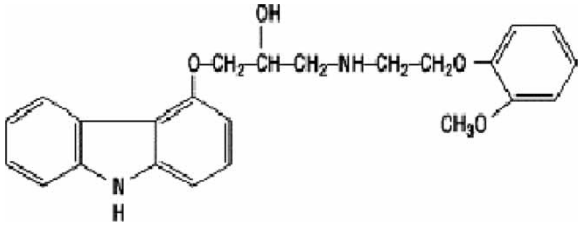

Figure 1. Carvedilol.

were made in methanol to produce working stock solutions of 5, 2, 1.5, 1, 0.5, 0.25, 0.1, and 0.05 μg/mL. These dilutions were used to spike serum in the preparation of calibration curves. The I.S working stock solution (2.5 μg/mL) was made from the primary stock solution using methanol for dilution. Calibration samples were prepared by spiking 1 mL of individual blank serum with the appropriate amount of drug on the day of analysis. Samples for the determination of recovery, precision, and accuracy were prepared by spiking control human serum in blanks of appropriate concentrations (5, 50, 100, and 500 ng/mL), and storing at −20°C.

Extraction Procedure

To 1 mL of serum, 100 μL of amitriptyline solution (2.5 μg/mL) was added and mixed well. To this, 300 μL of 0.1 N sodium hydroxide solution was added and vortexed for 1 min on a cyclomixer. Then, 5 mL of the solvent mixture (7:3 ratio of n-hexane, dichloromethane) was added and mixed for 5 min on a cyclomixer, followed by centrifugation at 3500 rpm for 10 min. The organic phase was separated and the residue was reextracted with 3 mL of the solvent mixture followed by centrifugation. The separated portions were pooled and subjected for evaporation in a vacuum oven. The residue was reconstituted in 100 μL of mobile phase and 20 μL of this solution was spiked on to the HPLC column.

Chromatographic Conditions

The HPLC system (Shimadzu, Japan) consisted of a LC−10AT solvent module, and a model SPD−10A, UV-Visible Spectrophotometric detector with LC 10 soft ware. The column used was a Kromasil KR 100-5C8 (stainless steel column of 25 cm length and internal diameter of 4.6 mm, packed with porous silicon spheres of 5 μ diameter). The mobile phase consisted of acetonitrile, 15 mM orthophosphoric acid (37:63), and 0.25 v/v% triethylamine mixture, and was adjusted to pH 2.5 with orthophosphoric acid. The elute was monitored at 238 nm with a flow rate of 1 mL/min. The sensitivity was set to 0.005 AUFS.

Linearity, Limit of Detection, and Limit of Quantification

The calibration samples were prepared by spiking 1 mL of control human serum with the appropriate amount of carvedilol and I.S on the day of analysis. The limit of detection (LOD) is the lowest level of analysis that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit of quantification (LOQ) was defined as the lowest concentration at which the RSD and deviation from the nominal concentration were less than 20%.

Assay Validation

The intra- and inter-run precision and accuracy of the assay (n = 5) were determined by percent C.V and percent error values, respectively, based on reported guidelines.[11] Samples containing 5, 50, 100, and 500 ng/mL concentrations were spiked for the determination of precision and accuracy. Samples were processed by appropriately spiking control human serum, to get concentrations of 5, 50, 100, and 500 ng/mL. At each concentration, 1 mL of serum was distributed into screw capped tubes and stored at −20°C. Five replicates at each concentration were processed as described in the sample preparation, on day 1, 3, 5, and 10 to determine intra day and inter day precision and accuracy. The percent error values were calculated by the following equation.

$$\text{Percent error} = \frac{(\text{Calculated concentration} - \text{Added concentration})}{\text{Added concentration}} \times 100$$

Extraction Efficiency

The efficiency of the extraction method to recover carvedilol and I.S from serum was tested using samples containing 5, 50, 100, and 500 ng/mL carvedilol and appropriate concentrations of I.S. These samples were then subjected to the sample preparation procedure explained above. The areas of carvedilol and I.S in the extracted samples (n = 3) were then compared with those of unextracted samples (n = 3) containing equivalent concentrations of carvedilol and I.S in the injection solutions.

Application to a Clinical Pharmacokinetic Study

The assay method was used to determine carvedilol concentrations in serum following oral administration of a carvedilol 6.25 mg tablet to 10 healthy male human volunteers after an over night fast. Blood samples (5 mL) were withdrawn from the ante cubital vein at the intervals of 0.5, 1, 2, 3, 4, 6, 8, 10, and 24 hrs following drug administration. The samples were allowed to clot and centrifuged at 3500 rpm for 10 min. The serum was separated and stored at −20°C until the commencement of analysis.

Pharmacokinetic parameters like peak serum concentration (C_{Max}), time to reach peak concentration (T_{Max}), area under the curve (AUC), and elimination half-life ($t_{1/2}$) for carvedilol were obtained for each subject using a computer program KINETICA (Innaphase corporation, 1999) meant for calculation of model independent parameters.

RESULTS AND DISCUSSION

Chromatography

A chromatogram of blank serum and serum sample obtained after 6 hrs of oral administration of a carvedilol tablet to one volunteer is shown in Figures 2 and 3. The analytical process of carvedilol and internal standard (IS) were resolved with good symmetry, the retention time of carvedilol and internal standard were 6.10 and 8.44 min, respectively (Figure 3). No endogenous interfering peaks were observed in the individual blank serum at the retention times of carvedilol and amitriptyline, thereby, confirming the specificity of the analytical method. System suitability parameters for the method were as follows: theoretical plates for carvedilol and I.S were 1686 and 5202, respectively. Tailing factor was less than 1.1 for both carvedilol and I.S and resolution between carvedilol and I.S was 3.6.

Quantification

The ratio of peak area of carvedilol to that of I.S was used for the quantification of carvedilol in serum samples. The calibration curves were linear in the concentration range 5–500 ng/mL. The calibration/regression equation is $y = mx + c$, where y represents the peak area ratio of carvedilol to I.S,

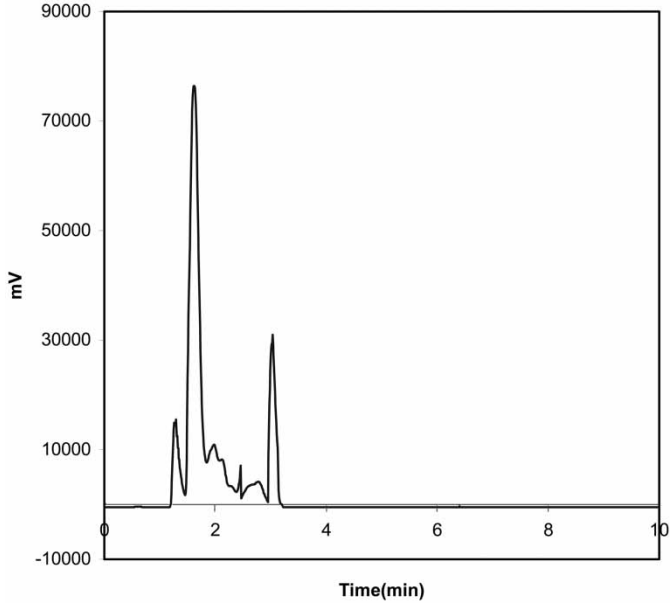

Figure 2. Typical HPLC chromatogram for analysis of carvedilol: blank serum.

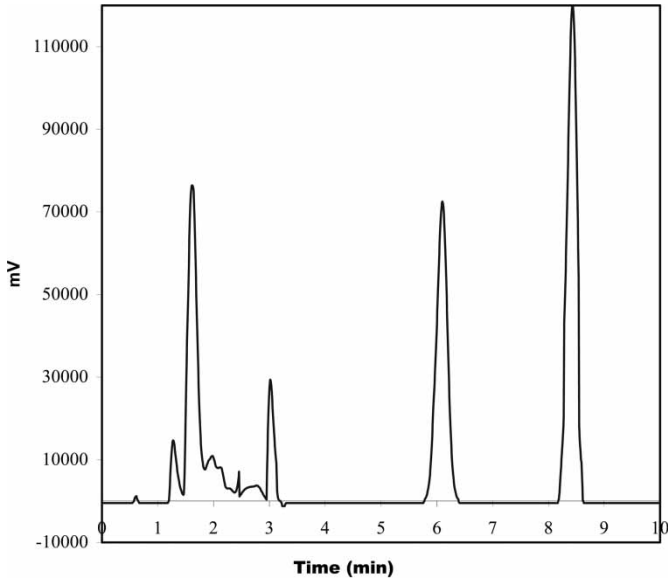

Figure 3. Typical HPLC chromatogram of carvedilol 6 h after dosing. Serum sample from a subject collected 6 h after dosing. Retention time of carvedilol is 6.10 min; amitriptyline is 8.44 min. The representative concentration was 24.70 ng/mL of carvedilol.

x represents the concentrations of carvedilol, m is slope of the curve, and c is the intercept. The equation of the calibration curve obtained from 6 points was $y = 0.0525x - 0.3204$ ($r^2 = 0.9979$).

The LOQ, established by determining the concentrations of four spiked calibration standards having a reproducibility with a relative standard deviation (RSD) less than 20% and an accuracy of 80 to 120%, was found to be 5 ng/mL and it was twice of the LOD. The LOD was 2.5 ng/mL with a relative standard deviation of 27%, the reproducibility was not observed with this concentration. As per the reported guidelines, the LOQ is approximately twice of the LOD.[12] Using this method, it is possible to further increase the sensitivity by increasing the serum/injection volume. The intra day precision of the assay was determined by analyzing five spiked serum samples at each concentration on the same day. For the determination of inter day precision, the samples were analyzed on five different days. The intra day and inter day coefficient of variation (% CV) and error (%) values are shown in Table 1. These values were within the limits (<15%) specified for inter and intra day precision.[13,14] The recovery of carvedilol from serum was estimated at 5, 50, 100, and 500 ng/mL concentrations. Serum samples (in triplicate) containing carvedilol and IS were extracted (1 mL serum with 10 mL of solvent mixture) and analyzed. Triplicate

Table 1. Intraday and Inter day accuracy and precision of the assay (n = 5)

Added conc (ng/mL)	Calculated conc (ng/mL)		CV (%)		Error (%)	
	Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
5	5.16	5.18	3.92	4.25	3.20	3.60
50	50.60	50.31	3.43	3.40	1.20	0.62
100	100.72	100.69	1.45	1.15	0.72	0.69
500	500.80	499.98	1.46	1.90	0.16	−0.004

samples containing similar concentrations of carvedilol in mobile phase were directly injected, and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure samples spiked with the same amount of carvedilol and proceeded similarly. The absolute recoveries ranged from 75–93% (Table 2). The accuracy of the method was verified by comparing the concentrations of carvedilol measured in extracted serum with the actual concentrations added.

The mean serum concentration vs time profile of carvedilol (up to 24 hrs) in 10 human volunteers following oral administration of carvedilol 6.25 is shown in Figure 4. A peak concentration of 27.94 ± 2.52 ng/mL (C_{max}, mean ± SD) for carvedilol was reached at 3.25 ± 0.46 hr ($t_{max,}$ mean ± SD). The half-life was found to be 11.05 ± 1.93 hr. Area under serum (AUC_{0-24}) concentration was found to be 272.72 ± 19.44 ng-hr/mL. These parameters were comparable with those reported earlier.[15]

CONCLUSIONS

These experiments confirm that the present method for the determination of carvedilol in human serum is simple, sensitive, specific, precise, accurate and require relatively small volumes of serum (1 mL). The calibration curve

Table 2. Recovery and accuracy of the proposed method

Conc ng/mL	Absolute recovery (%)		Accuracy (%)	
	Mean ± SD (n = 3)	Range (Min–Max)	Mean ± SD (n = 3)	Range (Min–Max)
5	75.92 ± 1.87	73.58−77.45	94.80 ± 1.56	93.5−96.3
50	87.16 ± 1.60	86.80−88.91	96.28 ± 1.24	95.4−97.7
100	91.24 ± 2.10	88.10−93.40	95.90 ± 2.81	92.9−98.5
500	93.11 ± 2.70	91.22−95.90	96.63 ± 2.52	94.2−99.3

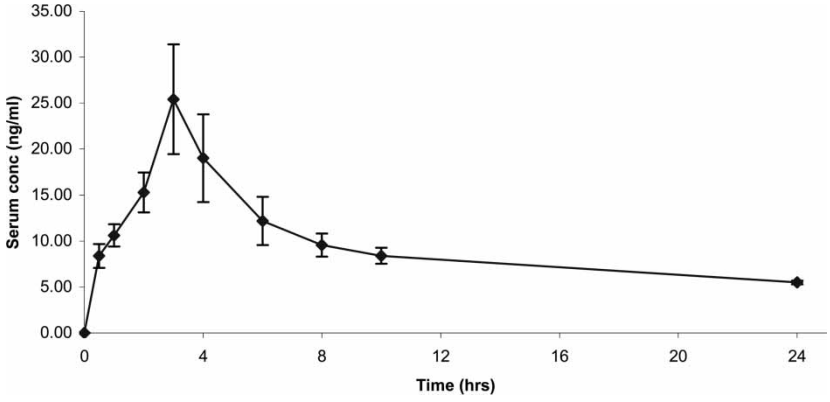

Figure 4. Serum concentration verses time profile of carvedilol after 6.25 mg oral administration. The data points are mean ± SD of 10 observations.

was linear in the concentration range between 5 and 500 ng/mL, hence, the method is suitable for conducting pharmacokinetic studies. There has been no report of measuring of carvedilol concentration by HPLC with UV detection. We therefore developed a new method for measuring the plasma concentration of carvedilol, which was sufficient for clinical use.

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