High-Performance Liquid Chromatography Determination of Carvedilol in Pig Serum

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Abstract

A simple and sensitive analytical method for quantification of carvedilol in pig serum was developed and validated. Carvedilol and internal standard (IS) were extracted into *n*-hexane–dichloromethane solvent system and separated using an isocratic mobile phase on a Phenomenex C_{18} column. The eluent was monitored by spectroflourimetric detector at a flow rate of 1.0 mL/min. The linearity range of proposed method was 1–1000 ng/mL. The intra-day and inter-day coefficient of variation and percent error values of the assay method were less than 15%, and mean recovery was more than 89.95 and 94.27 for carvedilol and IS, respectively. The method is applicable for use in the pharmacokinetic characterization of carvedilol after administration of buccal patch (6.25 mg) in pigs.

Introduction

Carvedilol [1-(9H-carbazol-4-yloxyl-3-[2-(2-methoxyphenoxy) ethylamino] propan-2-ol] (Figure 1A) is a non-cardioselective β-blocker that is used in the mild-to-moderate management of hypertension and angina pectoris (1,2). It is also used to reduce mortality in patients with left ventricular dysfunction following myocardial infarction. After oral administration, carvedilol is well-absorbed from the gastrointestinal tract with peak concentrations in plasma occurring about 1–2 h (2) but undergoes extensive first pass liver metabolism that results in an absolute bioavailability of about 25% (3).

Several chromatographic methods have been developed for the determination of carvedilol in biological samples, plasma (4–9), serum (10,11), urine (12,13), and cardiac tissue (14). Most of these methods are focused on the separation of enantiomers (7–9). However, these methods have various limitations, including column switching procedure (15), time-consuming sample clean-up, laborious extraction steps, low sensitivity, and long run times that are not suitable in all conditions. Two sensitive liquid chromatography–tandem mass spectrometry (LC–MS–MS) assays have been also reported for the stereo-selective analyses of carvedilol in plasma (16,17). Both the methods are very sensitive, having low quantitation limits. However, these methods are not suitable for routine pharmacokinetic purposes and bioequivalence studies due to using a 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.

None of the methods were reported for pig serum. There is a possibility of enhancement of bioavailability through the buccal route as it bypasses first pass metabolism. Moreover, carvedilol is a potent antihypertensive agent; it could cause sudden fall in blood pressure in human beings. Hence, the pre-clinical evaluation of new drug delivery systems like buccal patches in pigs, before testing in human beings, is more appropriate and advisable. Although several published methods are available for pharmacokinetic evaluation of carvedilol, very few reports are available in animal models (dogs) (18). In our investigation, pigs were selected as the animal model because their buccal membrane closely resembles the human buccal membrane in terms of structure and permeability (19). The present work was aimed at developing a sensitive high-performance liquid chromatography (HPLC) method for determination of carvedilol in pig serum. The advantages of present method include small sample volume, simple extraction procedure using inexpensive chemicals, and short run time. Protein precipitation was selected because it had obvious advantages such as shorter processing time, lesser organic solvent consumption, fewer steps, and good plasma sample clean-up. The mixture of *n*-hexane and dichloromethane was found to be the most suitable organic precipitant in the present study. We also demonstrate the applicability of this method for pharmacokinetic study in pigs.

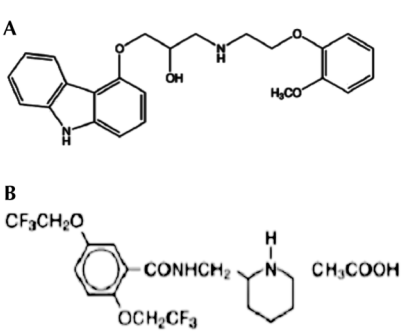

Figure 1. Structure of (A) carvedilol and (B) flecainide acetate.

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Experimental

Materials

Carvedilol and flecainide acetate pure samples were gifted by Sun Pharmaceuticals (Mumbai, India) and Ranbaxy Laboratories (Gurgaon, India), respectively. Acetonitrile and methanol (HPLC-grade) were obtained from Rankem (Delhi, India). Orthophosphoric acid was purchased from Merck (Mumbai, India) and triethylamine from SD Fine Chemicals (Mumbai, India). Both are AR-grade. Double-distilled water was used during the entire HPLC procedure.

Chromatographic conditions

The Shimadzu HPLC system (Kyoto, Japan) consisted of a LC-10AT solvent module, RF10AXL spectrofluorimetric detector with LC 10 software. The analytical column used was Phenomenex C_{18} column (250 mm × 4.6 mm i.d, particle size 5 µm) at ambient temperature (25°C). The mobile phase consists of acetonitrile, methanol, water, and triethylamine at a ratio of 25:20:54.9:0.1 (v/v). The pH was adjusted to 2.5 with orthophosphoric acid. The elute was monitored at 285 and 380 nm as excitation and emission wavelengths, respectively, at a flow rate of 1 mL/min. The injection volume was 20 µL, and detector sensitivity was set to medium (response 2).

Preparation of calibration standards and QC samples

The stock solutions of carvedilol and flecainide acetate were prepared in methanol at a concentration of 1.0 mg/mL each. The working solutions of 10 µg/mL and 500 ng/mL were prepared by appropriately diluting the stock solutions of carvedilol and flecainide acetate, respectively. Flecainide acetate was selected as an internal standard (IS) (Figure 1B) as its excitation and emission wavelengths were similar to that of carvedilol. Carvedilol working solution was used to prepare the spiking stock solutions for construction of eight-point calibration curve (1–1000 ng/mL) and quality control (QC) samples at four different levels (1.0, 50.0, 250.0, and 1000.0 ng/mL). All the stock solutions were refrigerated (4°C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 100 µL of respective spiking stock solutions to 300 µL of control pig serum and then aliquoted. These were stored at –20°C until analysis.

Sample preparation for analysis

Aliquot (300 µL) of the serum containing carvedilol was pipetted into microtubes, and 100 µL of IS (500 ng/mL of flecainide acetate) was added and vortexed to mix for 2 min. Sodium hydroxide (100 mM) of 150 µL was added, vortexed for 3 min, followed by the addition of 5 mL of solvent mixture (70:30) of *n*-hexane–dichloromethane. This was vortexed for 10 min and centrifuged at 3500 rpm. The supernatant (4.5 mL) was separated and allowed to evaporate in vacuum oven (Sheldon Manufacturing, Cornelius, OR) at 40°C. The evaporated residue was reconstituted with 100 µL of mobile phase, and 20 µL of the reconstituted sample was injected in to the HPLC system for analysis.

Table I. Composition of the Buccal Patch*

Formulation ingredient	Quantity
Carvedilol	334 mg
Hydroxy propyl methyl cellulose E15	2750 mg
Propylene glycol	412.5 µL
Solvent system (1:1 of dicloromethane–alcohol)	25 mL

*Note: The patch with an area of 1.27 cm^2 contains 6.25 mg of carvedilol.

Assay validation

The intra- and inter-day precision and accuracy of the assay were determined by percent coefficient of variation (CV) and percent relative error (RE) values, respectively, based on reported guidelines (20). Samples containing 1.0, 50.0, 250.0, and 1000.0 ng/mL concentrations were spiked for the determination of precision and accuracy. Five replicates at each concentration were processed as described in the "Sample preparation" section on day 1, 3, 5, and 10 to determine intra-day and inter-day precision and accuracy.

Limit of quantitation and limit of detection

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.

Extraction efficiency

The recovery of carvedilol was determined for QC samples at concentrations of 1.0, 50.0, 250.0, and 1000.0 ng/mL. Five replicates of each QC sample were extracted by the previously mentioned sample preparation and injected into the HPLC system.

Stability studies

To ensure the reliability of the results in relation to handling and storing of serum samples and stock standard solutions, stability studies were carried out at four different concentration levels (1.0, 50.0, 250.0, and 1000.0 ng/mL). Freeze-and-thaw stability for three cycles was determined at 1.0, 250.0, and 1000.0 ng/mL concentrations. The samples were obtained over three freeze-thaw cycles by thawing at room temperature for 2–8 h and then refreezing at –20°C for 12–24 h. The stability of spiked pig serum (1.0, 250.0, and 1000.0 ng/mL) stored at room temperature (bench top stability) was evaluated for 8 h and compared with freshly prepared extracted samples. The long-term stability of carvedilol at 1.0, 50.0, 250.0, and 1000.0 ng/mL in pig serum was assessed by carrying out the experiment after 30 days of storage at –20°C. The stock solution stability of carvedilol and IS were determined at room temperature for 12 h and upon refrigeration (4°C) for 14 days. The concentration of carvedilol after each storage period was related to the initial concentration as determined for the samples that were freshly prepared.

Application to pharmacokinetic study

The pharmacokinetic study was conducted in six male healthy

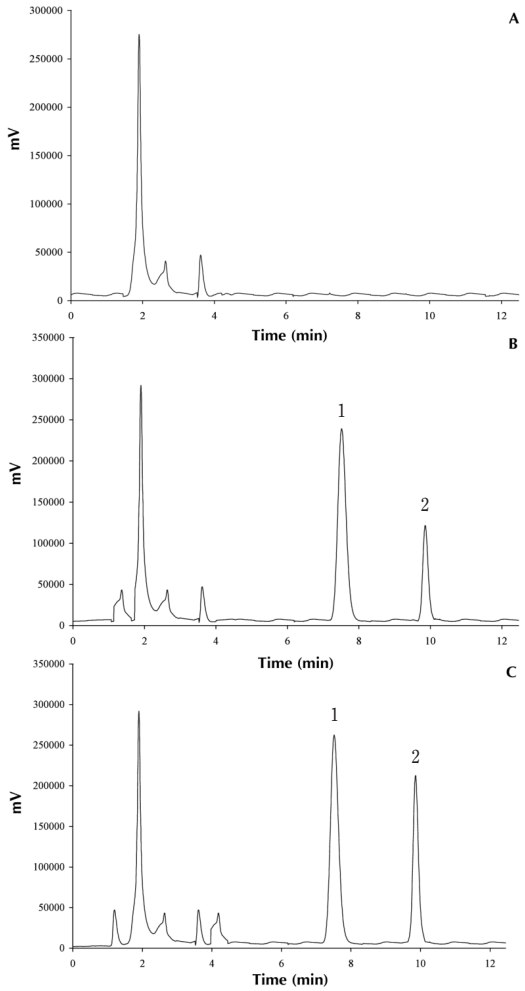

Figure 2. Typical HPLC chromatogram of carvedilol: (A) blank serum, (B) serum sample spiked with carvedilol (50 ng/mL), and (C) serum sample from a pig collected 10 h after administration of buccal patch; the corresponding carvedilol concentration was 87.49 ng/mL. 1 and 2 represent peaks of IS and carvedilol, respectively.

Table II. Intra-day and Inter-day Precision and Accuracy Data for Assay of Carvedilol in Pig Serum (*n* = 5)

Added conc. (ng/mL)	Calculated conc. (ng/mL)		%CV		% Error	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.0	1.02	1.18	7.82	12.53	2.000	4.000
50.0	50.80	49.25	7.48	9.27	1.600	–1.500
250.0	251.46	250.83	6.93	8.15	0.584	0.332
1000.0	1002.93	998.86	3.42	4.65	0.293	–0.114

Table III. Recovery and Accuracy of the Proposed Method

Conc. (ng/mL)	Absolute recovery			Accuracy (%)		
	Mean ± SD (*n* = 5)	Range (Min–Max)	% CV	Mean ± SD (*n* = 5)	Range (Min–Max)	% CV
1.0	89.95 ± 6.11	83.45–97.07	6.790	95.11 ± 9.46	84.56–104.97	9.950
50.0	91.89 ± 7.82	84.31–99.51	8.510	95.28 ± 6.49	88.05–102.87	6.810
250.0	95.26 ± 6.93	88.18–102.23	7.270	97.82 ± 5.18	91.15–103.62	5.300
1000.0	98.58 ± 3.26	94.45–103.11	3.310	99.28 ± 4.86	93.28 ± 104.92	0.490

pigs (body weight 25 ± 1.2 kg) with permission approved from the institutional ethical committee, University College of Pharmaceutical Sciences. The composition of buccal patch is shown in Table I. In brief, the buccal patch (6.25 mg per 1.27 cm^2) was placed in the buccal position of the oral cavity with the polymer side facing the mucosa of buccal cavity. A gentle pressure was applied for 1 min. In a crossover study, oral solution (6.25 mg in 5 mL of phosphate buffer pH 6.6 containing 20% v/v alcohol) was administered through a feeding tube. The crossover study was conducted after a wash period of 10 days. The blood samples (10.0 mL) were collected from the tail vein at intervals of 0.0, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 24.0 h. All blood samples were allowed to clot and were centrifuged for 10 min at 3000 rpm. The serum was separated and transferred into clean tubes and stored at –20°C until HPLC 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 pig using a computer program KINETICA (Thermo Scientific, Waltham, MA) meant for calculation of model independent parameters.

Results and Discussion

Chromatography

Typical chromatograms of pig blank serum, serum spiked with 50 ng/mL of carvedilol and 50 ng/mL of IS, and one pig serum sample obtained 10 h after administration of carvedilol buccal patch of (6.25 mg) are shown in Figure 2A–2C. The analytical process of carvedilol and IS was resolved with good symmetry, and the retention times of IS and carvedilol were 7.53 min and 9.88 min, respectively. No endogenous interfering peaks were observed in individual blank serum at the retention times of carvedilol and flecainide acetate, thereby confirming the specificity of the analytical method. System suitability parameters for the method were as follows: theoretical plates for carvedilol and IS were 1366 and 3644, respectively. Tailing factor was less than 1.1 for both carvedilol and IS, and the resolution between carvedilol and IS was 5.13.

Quantification and calibration curve

The ratio of peak area of carvedilol to that of IS was used for the quantification of carvedilol in serum samples. The calibration curve of 8 points was linear in the concentration range 1–1000 ng/mL with a correlation coefficient (*r*) of 0.9989.

Accuracy and precision

The accuracy and precision of the method were evaluated with QC samples at concentrations of 1.0, 50.0, 250.0, and 1000.0 ng/mL. The intra-day precision (expressed as percentage relative standard deviation, RSD) and accuracy (expressed as percentage RE) were determined by analysis of five replicates of QC samples at four different concentrations. The inter-day accuracy and precision were determined on four different days, and the results

are shown in Table II. The inter-day and intra-day precisions of the QC samples were satisfactory with RSDs less than 15% and accuracy with RE within ± 5%.

LOQ and LOD

LOQ was established by determining the concentrations of four spiked calibration standards. The LOQ of this method was found to be 1 ng/mL for carvedilol in pig serum with an RSD less than 20% and an accuracy of 80–120%. The LOD was determined to be 0.1 ng/mL based on a signal-to-noise (s/n) ratio of 3:1.

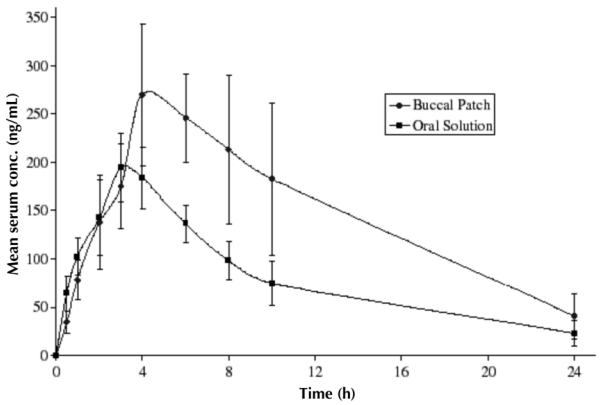

Figure 3. Mean (± SD) serum concentration-time profile of carvedilol in pig serum (n = 6) followed by the administration of oral solution (6.25 mg) and buccal patch (6.25 mg).

Table IV. Stability Study Results of Carvedilol

Stability	Spiked conc. (ng/mL)	Avg. calculated comparison sample conc. (ng/mL)	Avg. calculated stability sample conc. (ng/mL)	Avg. %
Bench-top*	1.0	1.08 ± 0.02	1.05 ± 0.03	97.22
	50.0	50.25 ± 0.08	50.15 ± 0.18	99.80
	250.0	251.29 ± 0.846	250.96 ± 1.22	99.87
Freeze and thaw†	1.0	1.06 ± 0.04	1.04 ± 0.03	98.11
	50.0	51.10 ± 1.20	50.59 ± 0.48	99.00
	250.0	250.48 ± 0.81	250.26 ± 0.83	99.91
Long-term‡	1.0	1.04 ± 0.02	1.01 ± 0.04	97.12
	50.0	50.16 ± 0.48	50.06 ± 0.24	99.80
	250.0	250.87 ± 0.82	250.50 ± 1.45	99.85
	1000.00	1001.09 ± 0.75	1000.29 ± 1.50	99.92

* After 8 h at room temperature. † After three freeze-thaw cycles.
‡ After 30 days at –20°C.

Table V. Pharmacokinetic Parameters of Carvedilol After Administration of Oral Solution and Buccal Patch to Pigs (n = 6)

Formulation	C_{Max} (ng/mL)	T_{Max} (h)	AUC_{0-n} (ng-h/mL)	AUC_{Total} (ng-h/mL)	$T_{1/2}$ (h)
Oral solution	207.08 ± 20.64	3.50 ± 0.55	287.51 ± 20.59	1813.20 ± 42.53	6.35 ± 2.41
Buccal patch	314.83 ± 31.46	5.66 ± 1.97	2340.58 ± 49.67	4154.37 ± 80.22	7.62 ± 1.04

Extraction recovery

The extraction recovery was determined by standard addition at four different concentrations (1.0, 50.0, 250.0, and 1000.0 ng/mL) for carvedilol and one concentration (50.0 ng/mL) for IS. The extraction recovery was calculated by comparing the peak areas of the prepared standard samples with those of the standard solutions. The results are shown in Table III. The extraction recovery of carvedilol at 1.0, 50.0, 250.0, and 1000.0 ng/mL was 89.95, 91.89, 95.26, and 98.58%, respectively. The mean recovery of flecainide acetate was found to be 94.27 ± 3.51%. The recovery of carvedilol using the described procedure was consistent and efficient.

Stability

Analysis of the stock solution was performed at 1000.0 ng/mL. After storage for 14 days at 4°C and at room temperature for 12 h, more than 98% of carvedilol remained unchanged, based on peak areas in comparison with freshly prepared solution of carvedilol (1000.0 ng/mL). This indicated that the carvedilol in standard solution was stable for at least 14 days when stored at 4°C and for 12 h at room temperature. Bench-top stability of carvedilol in serum was investigated at the concentrations of 1.0, 250.0, and 1000.0 ng/mL, and the results revealed that the carvedilol in serum was stable for at least 8 h at room temperature with average percentages of 97.22, 99.80, and 99.87%, respectively. The repeated freezing-thawing (three cycles) of serum samples spiked with carvedilol at three levels 1.0, 250.0, and 1000.0 ng/mL showed mean percentage concentrations of 98.11, 99.00, and 99.91%, respectively. Long-term stability of the carvedilol in serum at –20°C was also performed after 30 days of storage at four (1.0, 50.0, 250.0, and 1000.0 ng/mL) levels, which showed mean percentage concentrations of 97.12, 99.80, 99.85, and 99.92%, respectively. The results (Table IV) of the stability studies indicated that the carvedilol was stable in the studied conditions.

Application to pharmacokinetic study

The method was applied to the analysis of serum samples obtained after administration of carvedilol buccal patch (6.25 mg) and oral solution to six pigs. Figure 3 depicts the mean serum concentration-time curve of carvedilol. The pharmacokinetic parameters were estimated using the KINETICA software, and the results obtained were compared with that of oral solution (Table V). After administration of the buccal patch, a peak concentration of 314.83 ± 31.46 ng/mL (C_{Max}, mean ± SD) for carvedilol was reached at 5.66 ± 1.97 h (T_{Max}, mean ± SD). The half-life was found to be 6.35 ± 1.04 h. Area under serum concentration (AUC_{0-24}) was found to be 2340.58 ± 49.67 ng-h/mL. The bioavailability of carvedilol was improved significantly ($p < 0.05$) by administration of the buccal patch.

Conclusion

A simple, sensitive, and reliable method for the determination of carvedilol over the concentration range of 1–1000 ng/mL in pig serum by HPLC was

developed and validated. The method consisted of sample preparation by protein precipitation, followed by chromatographic separation and fluorescence detection. No interfering peaks were observed at the elution times of carvedilol and IS. The method was accurate, reproducible, specific, requires relatively small volumes of serum (300 µL), and is applicable to the evaluation of pharmacokinetic profiles of carvedilol in pigs. The developed HPLC method was found to be suitable for the analysis of carvedilol in pig serum. The bioavailability of carvedilol was found to be improved significantly ($p < 0.05$) after administration of the buccal patch. This method is also equally suitable for the estimation of carvedilol in human biological samples.

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