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Research Article

Validation and Optimization of a Simple RP-HPLC Method for Determination of Cilostazol in Human Serum

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ABSTRACT

Cilostazol is a synthetic antiplatelet and vasodilator agent. In the present study, a simple, sensitive and specific liquid chromatography (HPLC) method with UV detection was developed and validated for the quantification of cilostazol in human serum samples using diazepam as internal standard. The mobile phase consisted of water and acetronitrile (60:40%, V/V), pumped at a flow rate of 1.0 ml/min through the C-18 column at room temperature. Chromatographic separation was accompanied at 254 nm with a sensitivity of 0.0001. The developed method was selective and linear for cilostazol concentrations ranged in between 20 to 2000ng/ml for serum samples. The recovery of cilostazol ranged from 96.35 to 112.68%. The limit of quantitation (LOQ) of cilostazol was 20 ng/ml. The intraday accuracy ranged from 115.50% to 119.52%, the interday accuracy varied from 99.22 to 103.06%. For the LOQ, good values of precision (1.820 and 2.02% for intraday and interday, respectively) are also obtained. Acceptable results were obtained during stability study. This method proved to be simple, accurate and precise and can be used for pharmacokinetic and bioequivalence studies of cilostazol.

INTRODUCTION

Cilostazol [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone], is a potent inhibitor of platelet aggregation [1-2] possesses some vasodilator properties [3-4]. Clistazole (Figure-1) has been shown to inhibit platelet-derived growth factor (PDGF) secretion [5] and smooth muscle cell proliferation in vitro [5] and decrease triglyceride levels in vivo [6,7]. The antiplatelet activity is due to its cyclic-adenosine monophosphate (cAMP) phosphodiesterase (PDE)-inhibiting activity. The decrease in triglyceride levels is due to the inhibition of the production of 5'AMP from cAMP. Cilostazol has been approved by the US FDA for the management of intermittent claudication [8]. Cilostazol is recommended to be taken at a dose of 100 mg twice daily, 30 min before or 2 h after breakfast and dinner. It is rapidly absorbed after oral administration with peak plasma concentrations reaching at about 3 h after the dose. Co-administration of food increases the rate and extent of cilostazol absorption [9].

MATERIALS AND METHODS

Chemicals and reagents

The cilostazol standard and the internal standard diazepam were provided by Beximco Pharmaceuticals Ltd., Dhaka, Bangladesh. HPLC grade acetonitrile and methanol were procured (Merck, Germany) and water used was Milli-Q grade.

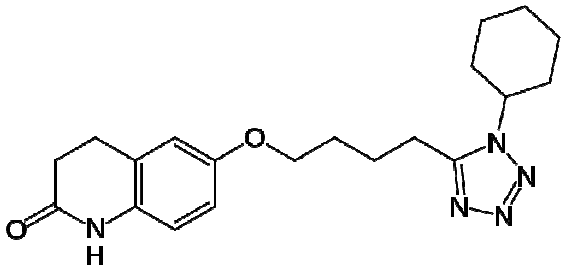

Figure 1: The Structure of cilostazol

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Instrumentation

A Shimadzu (Japan) HPLC system consisting of a SCL-10Avp system controller and two LC-8A pumps were used. Ultraviolet detection was achieved with a SPD-10Avp UV-VIS detector (Shimadzu, Japan). The drug analysis data were acquired and processed using LC solution (Version 1.03 SP3, Shimadzu, Japan) software running under Windows XP on a Pentium PC.

Chromatographic conditions

The mobile phase used was a mixture of water and acetonitrile (60:40, v/v), pumped at a flow rate of 1.0 ml/min through the column (C18; 5µ, 4.6 × 150 mm, Waters, USA) at room temperature. The mobile phase was degassed prior to use under vacuum by filtration through a 0.2µ nylon membrane. Concentrations were measured at 254 nm by UV detector at a sensitivity of 0.0001.

Preparation of calibration standards for serum sample assay

Stock solution of cilostazol was prepared having a concentration of 200 µg/ml in methanol. The stock solution was further diluted with acetronitrile to prepare the working standard solution having a concentration of 20 µg/ml. Diazepam (internal standard) stock solution (250 µg/ml) was prepared in methanol, from which a working standard solution of 20 µg/ml in diluent was prepared. Cilostazol calibration standards for serum were obtained by adding required amount of the working solution and 100 µl of diazepam (internal standard) solution (25 µg/ml) to drug free serum to achieve the cilostazol concentrations of 2000, 1000, 500, 100, 50, 20, ng/ml. Then they were vortexed for about 5 minutes for proper mixing and liquid-liquid extraction was performed by adding 2 ml methanol in 1 ml of each of the standard solutions. After vortex mixing, they were centrifuged at 10,000 rpm for 5 minutes and the supernatants were collected.

All these standards were stored at −30^{0}C until further analysis and analyzed by the above mentioned HPLC method for the construction of calibration curves and method validation. In all cases, the injection volume was 10 µl.

Preparation of QC samples

A series of quality control serum samples were prepared by adding blank serum with 100 µl of diazepam (internal standard) solution (25 µg/ml) and required amount of cilostazol to yield the final serum samples of 10, 100, 2000 ng/ml of cilostazol. These samples were then vortexed for 2 minutes and cilostazol was extracted according to method described in the previous section.

Method Validation

Calibration curves were constructed by using ratio of peak area of cilostazol to diazepam (internal standard) against the concentration of cilostazol and used to determine the cilostazol concentrations in unknown samples. The following parameters were determined for the validation of the analytical method developed for the determination of cilostazol in human serum-specificity, selectivity, linearity, range, precision, accuracy, limit of detection, limit of quantitation, recovery and stability [10-11].

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

A number of stationary and mobile phases were checked to determine the optimum separation and the highest analytical sensitivity for cilostazol and such sensitivity were given by the slope of calibration curves obtained for the assayed conditions. The optimal condition, with which the best result was obtained, has been reported in the experimental section. The mobile phase consisted of acetronitrile: water (60:40) to ensure the stability of cilostazol. The mean retention times for cilostazol and the internal standard were 8 min and 13 min respectively. The analysis time was set at 30 min. This time assured the elution of cilostazol and diazepam without any interference. Baseline resolution of the substances was achieved under the chromatographic conditions of the study. The suitability of the chromatographic system was checked daily before analysis by evaluating the tailing factor, the resolution and the system repeatability of three injections of a solution of cilostazol (100 ng/ml) and internal standard in mobile phase which demonstrated good performance of the chromatographic system throughout the study.

Selectivity

Drug-free human serum samples from six different sources were analyzed to ensure selectivity of the method [12]. These chromatograms were free of interferences at the retention times of cilostazol and the internal standard (Figure 2 A to C). The chromatograms for serum samples are shown in Figure D.

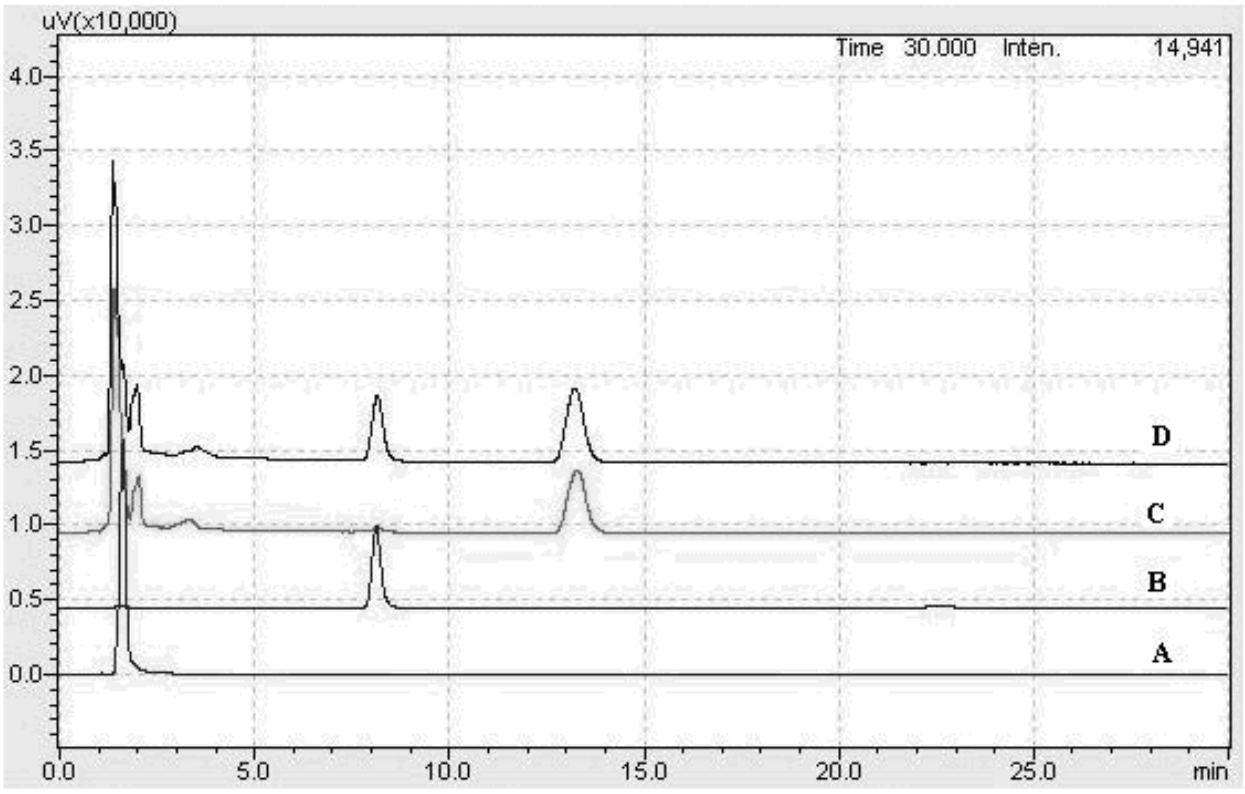

Figure 2: Representative chromatograms obtained from A) drug-free serum sample, B) serum with cilostazol, C) serum with internal standard and D) calibration standard for serum sample with cilostazol and internal standard

Sensitivity, Linearity and Range

According to IUPAC as cited in Roger Causon a method is said to be sensitive if small changes in concentration cause large changes in the response function [13]. Sensitivity can be expressed as the slope of the linear regression calibration curve, and it is measured at the same time with the linearity test. The sensitivity attainable with an analytical method depends on the nature of the analyte and the detection technique employed [14]. The linearity of the assay method was validated for serum (six standards) samples. The slope and the intercept of the calibration graphs were calculated through least squares by weighing linear regression of cilostazol to internal standard peak-area ratio. The concentration of cilostazol was studied over the range of 20 to 2000 ng/ml in serum. Data were fitted to the equation y = mx + b, where y is the peak area ratio, x is the drug concentration, m and b are the slope and y-axis intercept of the calibration curve, respectively. The calibration curve was found to be linear over the specified range. To construct a calibration curve in bioanalysis, a linear regression analysis is generally used. This analysis assumes invariant regression, implying that the residuals are minimized around the dependent variable (or response) and that the independent variable (or concentration) is errorless. Other assumptions involved are the independence and normal distribution of residuals as well as their homocedasticity (equal variances). Homocedasticity was not observed in our data, as usually occurs in bio-analysis [9, 15-16]. The mean regression parameters are given in Table 1. The mean (± S.D.) of the slope and intercept of the serum standards were 0.00030 (±0.00000) and 0.025 (±0.001), respectively. Coefficient of determination was greater than 0.99 on all calibration curves in serum.

Table 1: Regression data for the standard curve (n=6) of cilostazol in human serum

		Correlation coefficient	Slope	Intercept
Serum	Mean	0.994	0.00030	0.025
	S.D.	0.002	0.00000	0.001

Table 2: Intra-day and Inter-day precision and accuracy of cilostazol in serum (n=3)

Concentration (ng/ml)	Concentration found (ng/ml) Mean ± SD	Precision CV (%)	Accuracy (%)
Intra-day			
20	23.419 ± 0.4262	1.820	117.097
500	527.301 ± 20.2679	3.844	105.460
2000	2039.246 ± 10.4147	5.11	101.962
Inter-day			
20	20.15 ± 0.4068	2.02	100.75
500	566.322 ± 102.586	18.115	113.264
2000	2152.156 ± 341.2767	15.857	107.608

Table 3: Recovery of the bioanalytical method

Cilostazol recovery from serum samples (n = 3)			
Quantity added (ng/ml)	**Mean quantity obtained (ng/ml)**	**Recovery (%)**	**Average recovery (%)**
2000	2091.914	104.5967	104.813
500	527.379	105.4767	
20	20.87333	104.3667	

Table 4: Stability of cilostazol in serum QC samples in different storage conditions

Concentration (ng/ml)	Conditions	Mean concentration in serum (ng/ml)	Recovery in serum (%)
20	After 6 hr at ambient temperature	22.536	112.68
	48 hr storage at -40^0 C	19.270	96.35
	3 freeze thaw cycle	20.814	104.07
500	After 6 hr at ambient temperature	535.827	107.17
	48 hr storage at -40^0 C	538.201	107.64
	3 freeze thaw cycle	508.109	101.62
2000	After 6 hr at ambient temperature	2093.258	104.66
	48 hr storage at -40^0 C	2092.965	104.65
	3 freeze thaw cycle	2089.518	104.48

Limit of Quantitation (LOQ)

The lower limit of quantification can be defined as the lowest concentration on the calibration curve with acceptable precision and accuracy (CV<20%) (11). The lower limit of quantitation (LOQ) of cilostazol was found to be 20 ng/ml (precision 1.820% and 2.02% for intraday and interday, respectively).

Precision and accuracy

The precision of the method was assessed by determining the intra-day and inter-day coefficient of variation (CV) for different concentrations and are mentioned in the Table 2. The values obtained were in all cases lower than 14.14% for serum samples. The precision and accuracy of the method was found to be well within the limits considered as acceptable. Hence, the results indicate that the method is precise, accurate and reliable.

Recovery

The method of extraction of cilostazol and diazepam was evaluated for efficiency and the results are shown in the Table 3. The recovery of cilostazol was measured for all QC samples and the average recovery was found to be 104.813% from serum samples respectively. The method showed good efficiency in terms of recovery.

Stability

The stability of the analyte was evaluated for different under storage conditions of different temperatures. The results are depicted in the Table 4. For short term stability determination, stored serum was kept at ambient temperature for 6 hr exceeding that expected to be encountered during the routine sample preparation. Samples were pretreated and analyzed as above mentioned. The mean recoveries of the low, medium and high QC levels ranged from 96.35 to 107.64% for serum samples. These results indicated reliable stability behavior under the experimental condition of the regular runs.

Application of the method

A few HPLC-UV, LC-MS/MS, and GC-MS methods have been reported in different literatures. Some of these methods use complicated extraction instruments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction while other methods have a long turnaround time during analysis. To minimize these limitations, the present investigation provides a rapid, selective and sensitive HPLC-UV method that has short and simple extraction procedure, consume small amount of solvent and biological fluid for extraction with a short turnaround time. The validation data also indicates that the method is able to quantify the drug at low quantity like 6 ng/ml in biological samples with good precision and accuracy. For this we can be apply this method for the quantization of cilostazol in biological samples. In comparison with the previously developed methods, the present method offers an undoubted advantage in term of overall analytical performance.

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