Drug Development and Industrial Pharmacy, 33:173–179, 2007

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040600920366

informa
healthcare

A Validated Stability Indicating High Performance Reverse Phase Liquid Chromatographic Method for the Determination of Cilostazol in Bulk Drug Substance

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ABSTRACT A simple, rapid and accurate reverse phase high-performance liquid chromatographic (RP-HPLC) method was developed for the quantitative determination of cilostazol. The developed method is also applicable for the related substance determination in bulk drugs. The chromatographic separation was achieved on reversed-phase C-18 column. Eluents were monitored on photo-diode array detector at a wavelength of 210 nm using a mixture (50:50) of water and acetonitrile. Solution concentrations were quantified by external calibration. In the developed HPLC method, resolution between cilostazol and its potential impurities, namely Imp-A, Imp-B, and Imp-C were found greater than two. The drug was subjected to stress condition of hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was found to occur in alkaline medium stress condition. The developed RP-HPLC method was validated with respect to linearity, accuracy, precision, stability of analytical solutions, and robustness.

KEYWORDS Cilostazol, Validation, Forced degradation, RP-HPLC, Solution and mobile phase stability

INTRODUCTION

Cilostazol is a quinolinone derivative that inhibits cellular phosphodiesterase (more specific for phosphodiesterase III). It is an anti-platelet agent and is designated chemically as 6-[4-(1-cyclohexyl-1H-tetrazolyl-5-yl) butoxy]-3, 4-dihydro-2(1H)-quinoline. Hepatic cytochrome P-450 enzymes, mainly 3A4, extensively metabolize Cilostazol with metabolites largely excreted in urine. Cilostazol affects both vascular beds and cardiovascular function. Cilostazol could have pharmacodynamic interactions with other inhibitors of platelet function and pharmacokinetic interactions because of effects of other

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drugs on its metabolites by CYP3A4 or CYP2C19 (Physician desk reference, 2005). A literature survey revealed few high-performance liquid chromatographic (HPLC) methods for quantitative determination of Cilostazol and its metabolites in human plasma (Fu et al., 1999; Barmer et al., 2001), human urine (Tata et al., 2001) and human liver (Tata et al., 1998). Recently, a validated LC method was developed and reported (Ardhani et al., 2004) for the determination of Cilostazol in tablets. Apparently no stability indicating analytical method for Cilostazol is available in literature and it was felt necessary to develop a stability indicating LC method for determination and quantitative estimation of Cilostazol.

This paper describes the assay and validation method for accurate quantification of Cilostazol and all three impurities in bulk samples, respectively.

MATERIALS AND METHODS

Instrumentation

Quantitative HPLC analysis was performed on a high-performance liquid chromatography which consisted of a dual piston reciprocating two LC-10AT VP pumps (model HPLC class 10AT), photo-diode array detector (model SPD-10M VP) and auto sampler of SCL-10A series from Shimadzu, Japan. The HPLC system was also equipped with data acquisition and processing software "Class-LC-10 series" from Shimadzu, Japan.

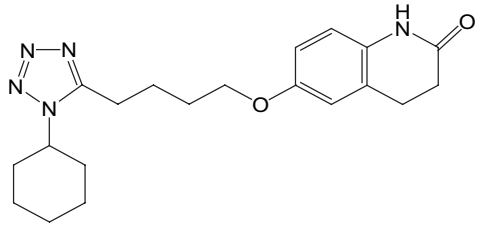
Materials, Reagents and Chemicals

Samples of Cilostazol and its three potential impurities namely Imp-A, Imp-B, and Imp-C (Fig. 1) were received from Wockhardt Pharmaceuticals Ltd, Aurangabad, India. The purity of the standard cilostazol sample was 99.8% while the purity of Imp-A, Imp-B, and Imp-C was 99.1, 98.85, and 98.3%, respectively. HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Pure water was prepared by using Millipore Milli Q Plus Purification System (Bedford, MA, USA).

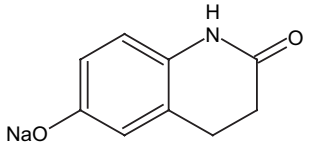
Chromatographic Conditions

The experiment was performed on a Inertsil C-18 stainless steel column having 250 mm×4.6 mm (ID) and 5 µm particle size. The mobile phase consisted of 50% acetonitrile and 50% water. The mobile phase

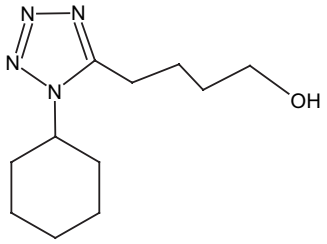
6-[4-(1-cyclohexyl-1H-tetrazolyl-5-yl) butoxy]-3,4-dihydro-2 (1H)-quinoline **(cilostazol)**



6-hydroxy-3, 4-dihydrocarbostyril-sodium salt **(Imp-A)**



5-(4-hydroxybutyl)-1-cyclohexyl-1***H***-tetrazole **(Imp-B)**



1-cyclohexyl 1-5-[4, 4bis (3, 4-dihydro-1H-quinolin-2-one-6-yloxy) butyl]-1-H-tetrazol **(Imp-C)**

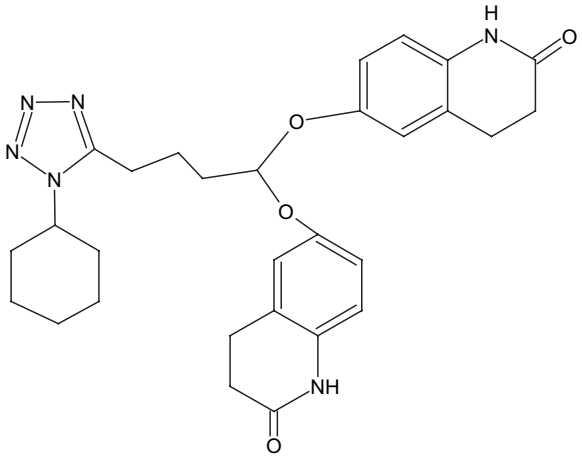


FIGURE 1 **Chemical structures of cilostazol, Imp-A, Imp-B, and Imp-C.**

was filtered through a nylon membrane (pore size 0.45 μm) and degassed with a helium spurge for 10 min. The chromatography was performed at room temperature using a flow rate of 1 mL/min. The run time was set to 15 min. The column temperature was maintained at 30°C and eluents were monitored on photodiode array detector at a wavelength of 210 nm. The volume of each injection was 20 μL.

Preparation of Standard Solutions

A stock solution of Cilostazol (1 mg/mL) was prepared by dissolving appropriate amount in mobile phase. Working solutions of 500 and 100 μg/mL were prepared from above stock solution for determination of related substances and assay, respectively. A stock solution of impurities (mixture of Imp-A, Imp-B, Imp-C) at 0.5 mg/mL was also prepared in mobile phase.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (ICH, 2000). Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.

The specificity of the developed LC method for cilostazol was determined in the presence of its impurities, namely Imp-A, Imp-B, Imp-C, and degradation products. Forced degradation studies were also performed on cilostazol to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study included light (performed as per ICH Q1B), heat (60°C), acid hydrolysis (0.1 N HCl), alkali hydrolysis (0.1 N NaOH), water hydrolysis and peroxide hydrolysis (0.01% H_2O_2). For heat and light studies, study period was 10 days whereas for acid, base, water hydrolysis, and oxidation, it was 48 hr. Shimadzu SPD-10M VP photo-diode array detector was used to check peak purity of stress samples. The purity factor within the threshold limit obtained in all stressed samples demonstrated the analyte peak homogeneity. Assay studies were performed for stress samples against qualified reference standard. Assay also calculated for bulk sample by spiking all three impurities (Imp-A, Imp-B, and Imp-C) at the specification level (i.e., 0.2% of the analyte concentration which is 500 μg/mL).

Precision

Six independent assays of the test sample of cilostazol against qualified reference standard were performed and. the percentage of relative standard deviation of six assay values obtained was calculated.

The precision of the related substance method was checked by injecting six individual preparations of (0.5 mg/mL) cilostazol spiked with 0.2% each of Imp-A, Imp-B, and Imp-C with respect to analyte concentration. The percentage of RSD of area for each Imp-A, Imp-B, and Imp-C was calculated.

The intermediate precision of the method was also evaluated using different analyte and a different instrument in the same laboratory.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for Imp-A, Imp-B, and Imp-C were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentration (ICH, 1995). Precision study was also performed at the LOQ level by injecting six individual preparations of Imp-A, Imp-B, and Imp-C and calculating the percentage of RSD of the area.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three-concentration levels, that is, 50, 100, and 150 μg/mL in bulk drug sample. The percentage of recoveries was calculated from slope and Y-intercept of the calibration curve obtained. Accuracy/recovery experiments were performed in triplicate.

The bulk sample provided by Wockhardt Limited had no Imp-A, Imp-B, and Imp-C. Standard addition and recovery experiments were conducted to determine accuracy of the related substance method for the quantification of all three impurities in bulk drug samples.

The study was performed in triplicate at 0.075, 0.15, and 0.225 of the analyte concentration (500 μg/mL). The percentage of recoveries for Imp-A, Imp-B, and Imp-C were calculated from the slope and Y-intercept of the calibration curve obtained.

Linearity

Linearity test solutions for assay method were prepared from stock solution at six concentration levels from 25–150% of assay analyte concentration (25, 50, 75, 100, 125, and 150 μg/mL). The peak area versus concentration data was performed by least-squares linear regression analysis.

Linearity test solutions for related substance method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 200% (1.5 μg/mL) of the specification level (LOQ, 0.075, 0.15, 0.187, 0.225, and 0.3%).

Linearity test was performed for three consecutive days in same concentration range for both assay and related substance method. The percentage of RSD value of the slope and Y-intercept of the calibration curve was calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were purposely altered and the resolution between cilostazol, Imp-A, Imp-B, and Imp-C was evaluated.

The flow rate of the mobile phase was 1 mL/min. To study the effect of flow rate on the resolution, it was changed by 0.2 units from 0.8 to 1.2 mL/min. The effect of the column temperature on resolution was studied at 25 and 35°C instead of 30°C. The effect of the percent organic strength on resolution was studied by varying acetonitrile by –3 to +3% while other mobile phase components were kept constant.

Solution Stability and Mobile Phase Stability

The solution stability of cilostazol in the assay method was performed by leaving both the test solutions of the sample and reference standard in tightly capped volumetric flasks at room temperature for 48 hr. The same sample solutions were assayed for 6 hr interval for the study period. The solution stability and mobile phase stability were also carried out by assaying the freshly prepared sample solution against freshly prepared reference standard solution for 6 hrs. interval up to 48 hrs. The percentage of RSD in cilostazol assay was calculated for the study period during mobile phase and solution stability experiments.

The solution stability of cilostazol and its impurities in the related substance method was performed by leaving spiked sample solution in tightly capped volumetric flask at room temperature for 48 hr. Contents of Imp-A, Imp-B, and Imp-C were determined for the study period after every 6 hr. Mobile phase stability was also performed for 48 hr by injecting the freshly prepared sample solutions every 6 hr. Content of Imp-A, Imp-B, and Imp-C were checked in the test solutions.

RESULTS AND DISCUSSION

Method Development and Optimization

The main target of the chromatographic method was to get the separation of critical closely eluting impurities, namely Imp-A, Imp-B, and Imp-C. Impurities were co-eluting by using different stationary phases like C18, C8, phenyl, and cyano and different mobile phases. The chromatographic separation was achieved on an Inertsil C-18, 250 mm × 4.6 mm, 5 μm column by using acetonitrile and water as mobile phase. The flow rate of the mobile phase was 1 mL/min. At 30°C column temperature, the peak shape of cilostazol was found symmetrical. In optimized conditions cilostazol, Imp-A, Imp-B, and Imp-C were well separated with resolution greater than two and typical retention times of Imp-A, Imp-B, Imp-C and cilostazol were about 2.8, 4.4, 7.2, and 8.3 min, respectively. ClogP as calculated on "Accord for excel software" for cilostazol was 3.75 and for Imp-C it was 4.86 with reference to 100% water, which means logP of Imp-C was higher than that of parent drug. But Imp-C elutes earlier than cilostazol, which may be because of additional amide function in Imp-C and there would be decrease in the value of pKa because of the addition of mobile phase.The system suitability results are given in Table 1 and the developed LC method was found to be

TABLE 1 System Suitability Parameter

Compound (n = 3)	USP resolution	USP tailing factor (R_s)	No. of theoretical plates (N) USP tangent method
Imp-A	–	1.5	3664
Imp-B	7.53	1.4	5690
Imp-C	9.42	1.1	6313
Cilostazol	2.95	1.2	7884

TABLE 2 Summary of Forced Degradation Study

Stress condition	Degradation time	Assay of cilostazol (%)	Remarks
Acid hydrolysis (0.1 N HCl) formed	48 hr	98.95	No degradation products
Base hydrolysis (0.1 N NaOH)	48 hr	93.31	Unknown degradation product at retention time 9.90 min.
Oxidation (0.01% H_2O_2)	48 hr	98.83	No degradation products formed
Thermal (60°C)	10 days	99.32	No degradation products formed
Light (photolytic degradation)	10 days	99.54	No degradation products formed

specific for cilostazol and its three impurities, namely Imp-A, Imp-B, and Imp-C (Table 2).

Precision

The RSD of assay of cilostazol during assay method precision study was within 1% and the RSD of area of Imp-A, Imp-B, and Imp-C in related substance method precision study were within 3%. The RSD of assay results obtained in intermediate precision study was within 0.7% and the RSD of area of Imp-A, Imp-B, and Imp-C were well within 3%, conforming good precision of the method.

Limit of Detection and Limit of Quantification

The limit of detection of Imp-A, Imp-B, and Imp-C were 0.02, 0.03 and 0.02% of analyte concentration (i.e., 500 µg/mL) for 20 µL injection volume. The limit of quantification of Imp-A, Imp-B, and Imp-C were 0.06, 0.09, and 0.06% of analyte concentration (i.e., 500 µg/mL) for 20 µL injection volume. The precision at LOQ concentration for Imp-A, Imp-B, and Imp-C were below 4%.

Accuracy

The recovery of cilostazol in bulk drug samples ranged from 99–101.5% (Table 3). The percentage recovery of Imp-A, Imp-B, and Imp-C in bulk samples ranged from 96.5–103.2. HPLC chromatogram of pure (unspiked) sample is shown in Fig. 2a and spiked samples at 0.2% level of all three impurities in cilostazol bulk sample are shown in Fig. 2b.

Linearity

Linearity calibration plot for assay method was obtained over the calibration ranges tested, that is, 25–150 µg/mL with the linear regression equation $y = 4223.503\ X + 6433.0$ and correlation coefficient of $R^2 = 0.9997$ was obtained. Linearity was checked for assay method over the same concentration range for three consecutive days. The percentage of RSD values of the slope and Y-intercept of the calibration curves

TABLE 3 Recovery Results of in Bulk Sample

Sr. no.	Added (µg) (n = 3)	Recovered (µg)	Recovery (%)	RSD (%)
1	51	50.5	99.02	0.7
2	101	101.7	100.69	0.5
3	153	155.3	101.50	0.8

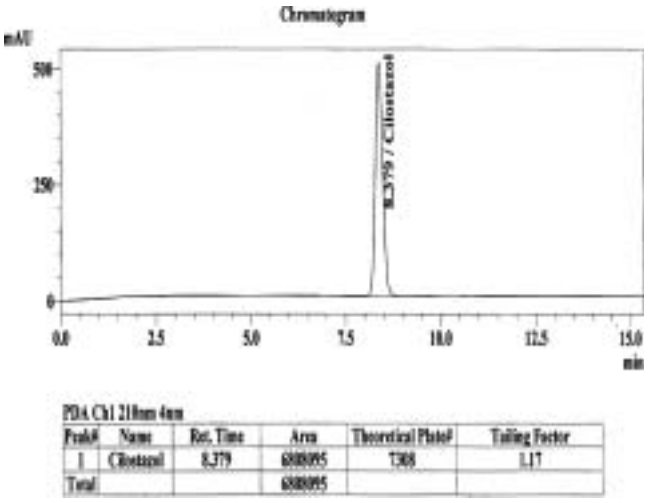
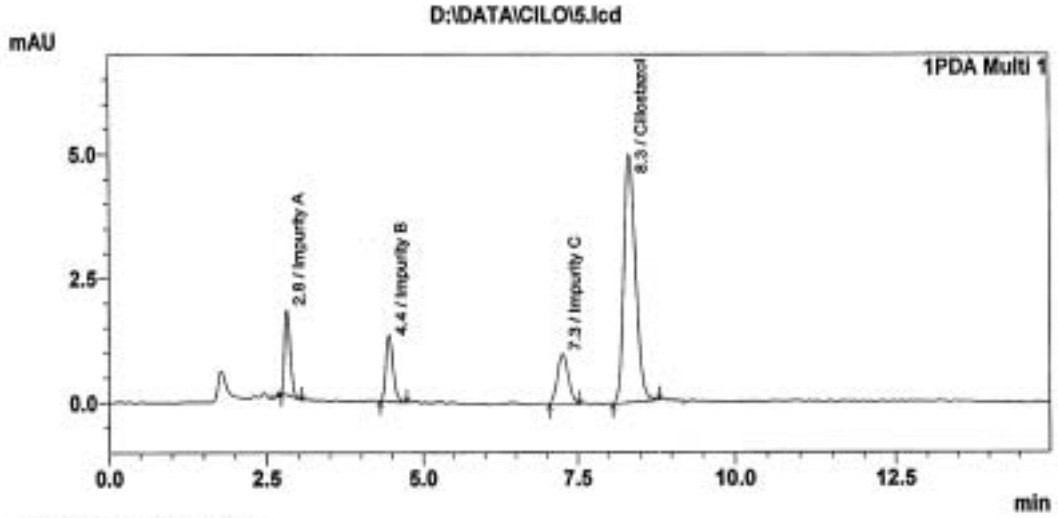


FIGURE 2A Typical HPLC chromatogram of pure (unspiked) bulk sample.



PDA Ch1 210nm 4nm

Peak#	Name	Ret. Time	Area	Area %	Resolution	Theoretical Plate#	Tailing Factor
1	Impurity A	2.816	10338	10.605	0.00	3652	1.56
2	Impurity B	4.447	10533	10.806	7.73	5693	1.38
3	Impurity C	7.257	12424	12.745	9.35	6316	1.15
4	Cilostazol	8.335	64184	65.844	2.91	7876	1.18

FIGURE 2B HPLC chromatogram of spiked Imp-A, Imp-B and Imp-C in pure cilostazol sample.

TABLE 4 Linearity Data of Cilostazol Sample

No.	Concentration injected (μg/mL)	Average peak area	RSD (%)
1	25	116973	0.760
2	50	212946	0.740
3	75	321920	0.510
4	100	427893	0.443
5	125	533865	0.543
6	150	642340	0.970

were 1.7 and 2.5, respectively. The linearity results are given in Table 4. The results show that an excellent correlation existed between the peak area and concentration of the analyte.

Linear calibration plot for related substance method was obtained over the calibration ranges tested, that is, LOQ 0.06–0.3% for Imp-A, Imp-B, and Imp-C. The correlation coefficient obtained was greater than 0.996. Linearity was checked for related substance method over the same concentration range for three consecutive days. The percentage of RSD values of the slope and Y-intercept of the calibration curves were 3.3 and 3.9, respectively. The results show that an excellent correlation existed between the peak area and concentration of Imp-A, Imp-B and Imp-C.

Robustness

In all the deliberately varied chromatographic conditions (flow rate, column temperature and organic solvent), the resolution between closely eluting impurities, namely Imp-A, Imp-B, and Imp-C was greater than 2.0, illustrating the robustness of the method (Table 5).

Solution Stability and Mobile Phase Stability

The RSD of assay of cilostazol during solution stability experiments was within 1%. No significant changes were observed in the content of Imp-A, Imp-B, and Imp-C during solution stability and mobile phase experiments using related substances method. The solution stability and mobile phase stability experimental data confirms that sample solutions and mobile phase used during assay and related substance determination were stable up to 48 hr.

TABLE 5 Results of Robustness Study

Sr. no.	Parameter	Variation	Resolution between Imp-C and cilostazol
1	Temperature (±5°C of set temperature)	(a) At 20°C	2.99
		(b) At 30°C	2.90
2	Flow rate (±20% of the set flow)	(a) At 0.8 mL/min	3.02
		(b) At 1.2 mL/min	2.87
3	Organic solvent (±3%)	(a) At +3% organic solvent	2.71
		(b) At –3% organic solvent	2.97

Results of Forced Degradation Studies

Degradation was not observed in cilostazol stressed samples that were subjected to light, heat, acid, peroxide, and water hydrolysis. The degradation of drug substance was observed under base hydrolysis. Cilostazol was degraded during base hydrolysis (in 0.1 N NaOH after 48 hr treatment) leads to the formation of unknown degradation peak at relative retention time 1.18 min (Fig. 3). Peak purity test results derived from SPD-10M VP, confirmed that the cilostazol peak was homogenous and pure in all the analyzed stress samples. The assay of cilostazol was unaffected in the presence of Imp-A, Imp-B, Imp-C, and its degradation products confirmed the stability, thus indicating power of the developed method.

CONCLUSION

The reverse phase high performance liquid chromatographic (RP-HPLC) method was developed for quantitative and related substance determination of cilostazol in bulk drug substance that was precise, accurate, rapid, and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis and to check the stability of bulk samples of cilostazol. The developed method also can be conveniently used for the assay determination of cilostazol in formulation.

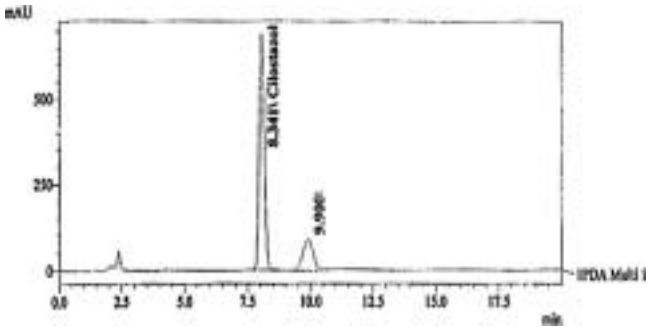


FIGURE 3 HPLC chromatogram of cilostazol under alkaline stress condition (0.1 N NaOH after 48 hr treatment).

AKNOWLEDGMENTS

We would like to thank management of Wockhardt Pharmaceuticals Ltd, Aurangabad, India for donation of drugs.

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