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Quantitative estimation of cilostazole in rat plasma by HPLC-ms/ms and its application to pharmacokinetic study

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ABSTRACT

Authors developed a simple, highly sensitive, selective, rapid, rugged and reproducible liquid chromatography–tandem mass spectrometry method for the quantification of Cilostazole (CZ) in rat plasma using Cilostazole d4(CZIS) as an internal standard (IS). Chromatographic separation was performed on Princeton Spher-100 C18, 50 × 4.6 mm, 5 µm column. Mobile phase composed of 0.1% formic acid: acetonitrile (40:60 v/v), with 0.6 mL/min flow-rate. Drug and IS were extracted by Liquid- liquid extraction. CZ and CZIS were detected with proton adducts at m/z 370.2→124.9 and 374.3→125.1 in multiple reaction monitoring (MRM) positive mode respectively. The method was validated with the correlation coefficients of (r^2) e" 0.9950 over a linear concentration range of 0.5-1500.0 ng/mL. This method demonstrated intra and inter-day precision within 1.00 to 3.73 and 1.25 to 4.45 % and accuracy within 97.33 to 100.16 and 92.0 to 99.42 % for CZ. This method is successfully applied in the Pharmacokinetic study of male rats.

KEYWORDS

Liquid chromatography;
Mass spectrometry;
Cilostazole;
Rat plasma;
Pharmacokinetic study.

INTRODUCTION

Cilostazol (6-[4-(1-cyclohexyl-1H-1, 2, 3, 4-tetrazol-5-yl)butoxy]-1,2,3,4-tetrahydroquinolin-2-one) is a quinolinone derivative that inhibits cellular phosphodiesterase used in the management of some peripheral vascular disorders. Its molecular weight is 369.46 and its empirical formula is $C_{20}H_{27}N_5O_2$.[1].(Figure1). Cilostazol is absorbed after oral administration. A high fat meal increases absorption, with an approximately 90% increase in C_{max} and a 25% increase in AUC. Absolute bioavailability is not known. Protein binding is 95-98%. Cilostazol is extensively metabolized by hepatic cytochrome P-450 enzymes, mainly 3A4, and, to a lesser extent, 2C19, with metabolites largely excreted in urine. Cilostazol has two active metabolites (3, 4-dehydrocilostazol and 4-trans-hydroxy-cilostazol), which account for approximately 50% of its effects on inhibiting phosphodiesterase III. Its pharmokinetics are dose proportional and its active metabolites have elimination half-lives of approximately 11-13 hours. Cilostazol is eliminated predominately by metabolism and subsequent urinary excretion of metabolites. The primary route of elimination was via the urine (74%), with the remainder excreted in feces (20%)[2-7].

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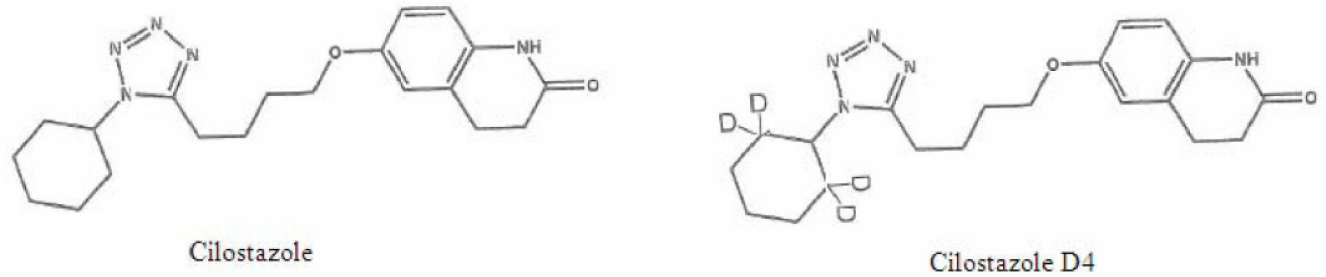

Figure 1 : Chemical structures of Cilostazole and Cilostazole D4

Literature survey reveals that several methods were reported for quantification of Cilostazole in Human plasma by LC-MS[8,9], Rat plasma by LC-MS[10], Human urine by HPLC[12], Human plasma by HPLC[13-15],Tablet dosage form by HPLC[16], Stability studies by HPLC[17], Bulk dosage form by HPLC[18]. Among all, LC-MS methods in Human plasma [8,9], LC-MS by Rat plasma[10] achieved best results in terms of high sensitivity and rapid analysis time.

Nirogi, et.al[8] developed the method by LC and LC-MS in human plasma with the linearity range 5-2000 ng/mL, run time with 2.5 min, mosapride as an internal standard. Bramer, et.al[9] published 'Method for the quantitative analysis of cilostazol and its metabolites in human plasma using LC/MS/MS'. Using this technique the procedure was observed to be specific and linear over the range 5-2000 ng/ml,with long run time 17.5minutes and less plasma volume (50 µL) usage. Varanasi et.al[10], developed a method by using LC-MS/MS in Rat plasma. In this method they quantified 20 to 2000 ng/ mL with short run time 3.5 minutes, and less plasma volume (50 µL) usage, and used repaglinide as an internal standard.

Among all developed methods Nirogi. et.al[8] achieved high sensitive with 5 ng/ml, Rapid method at 2.5 min RT. However they have not used deuterated or analogue based internal standard, and used large amount of plasma sample (100 µL).

The proposed method covers all the drawbacks to develop and validate the most simple, highly sensitive (10 times higher sensitive than Nirogi.et.al., selective, rapid (2.5 min run time), rugged and reproducible analytical method for quantitative determination of CZ in rat plasma by LC-MS/MS with a small amount of sample volume (50 µL) and used cilostazole-d4 as an internal standard. The developed method would be applied in pharmacokinetic study of different formulations.

MATERIALS AND METHODS

Chemicals and reagents

Cilostazole and Cilostazole D4 Obtained from Creative Organics, Bangalore. HPLC grade methanol, Acetonitrile purchased from Jt. Baker Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA. Formic acid was purchased from S. D fine chemicals Mumbai. Sodium carbonate anhydrous, Methyl t-butyl ether was purchased from Merck speciality private limited, worli, Mumbai. Ultra pure water from Milli-Q system (Millipore, Bedford, MA, USA) was used through the study. All other chemicals in this study were of analytical grade. Rats and rat plasma was obtained from Bioneeds, Bangalore.

Instrumentation

HPLC system (1200 Series Agilent Technologies, Germany) connected with triple quadrupole mass spectrometer instrument (API 4000, Toronto, Canada). Data processing was performed with the Analyst 1.4.1 software package (SCIEX). Ionization was performed by Electro spray positive mode with Unit Resolution.

Detection

Mass parameters were optimized to get the product ions of m/z: 124.9, m/z: 125.1 from its respective precursor ions of CZ $[M+H]^+$ (m/z: 370.2) and CZIS $[M+H]^+$ (m/z: 374.3) with Source temperature 500 °C, Ion Spray voltage 5500 volts, Heater gas, Nebulizer gas 35 psi each, Curtain gas 25 psi, CAD gas 4 psi, (all gas channels with nitrogen) Source flow rate 600 µL/ min without split, Entrance potential 10 V, Declustering potential 65V for analyte and 75V for internal standard, Collision energy 35 V for both analyte and internal standard, Collision cell exit potential 14 V for analyte

and 16 V for internal standard.

Chromatographic conditions

Chromatography was performed on Princeton Spher-100 C18, 50 × 4.6 mm, 5 µm analytical column at 40°C, with 0.1% formic acid: acetonitrile (40:60 v/v) as mobile phase at a flow rate of 0.6 mL/min. CZIS was used as an internal standard in terms of chromatography and extractability. The drug and internal standard was eluted at 1.39 ± 0.2 min and 1.38 ± 0.2 min with 2.5 min total run time.

Preparation of standards and quality control (QC) samples

Standard stock solutions of CZ (250.0µg/mL) and CZIS (100.0µg/mL) was prepared in methanol. The internal standard spiking solution (100.0 ng/mL) was prepared in 50% methanol from CZIS standard stock solution (100.0µg/mL). Standard stock solutions and Internal standard spiking solutions were stored in refrigerator conditions (2-8 °C) until analysis. Standard stock solution of CZ was added to screened drug-free rat plasma to obtain concentration levels of 0.5, 1.0, 5.0, 10.0, 75.0, 150.0, 300.0, 600.0,900.0,1200.0 and 1500.0 ng/mL for analytical standards and 0.5, 1.5, 750.0, 1000.0 ng/mL for Quality control standards and stored in a -30°C freezer until analysis. Respective aqueous standards were prepared in reconstitution solution (Acetonitrile 0.1 % formic acid (60:40) and stored in refrigerator conditions 2-8 °C until analysis.

Sample preparation

Liquid-liquid extraction was used to isolate drug and IS from rat plasma. For this purpose, 100µL of IS (100.0 ng/mL) and 50 µL of plasma sample (respective concentration) was added into labeled polypropylene tubes and vortexed briefly. Followed by, 500 µL of 0.5N sodium carbonate, 3.0 mL of extraction solvent (methyl tertiary butyle ether) were added and vortexed for 15 min. Then the samples were centrifuged at 4000 rpm for 5 min at 20°C temperature. Subsequently, the supernatant from each sample was transferred into respective polypropylene tubes. After that, all the samples were kept for evaporation under nitrogen at 40°C. The dried residue was reconstituted with 500 µL of reconstitution solution and vortexed briefly. Finally, the extracted sample was transferred into auto sampler vials and injected into LC-MS/MS.

Selectivity and specificity

The selectivity of the method was determined by six different rat blank plasma samples, which were pretreated and analyzed to test the potential interferences of endogenous compounds co-eluting with analyte and IS. Chromatographic peaks of analyte and IS were identified based on their retention times and MRM responses. The peak area of CZ at the respective retention time in blank samples should not be more than 20% of the mean peak area of LOQ of CZ. Similarly, the peak area of CZIS at the respective retention time in blank samples should not be more than 5% of the mean peak area of LOQ of CZIS.

Recovery

The extraction recovery of CZ and CZIS from rat plasma was determined by analyzing quality control samples. Recovery at three concentrations (1.5, 750.0, and 1000.0 ng/mL) was determined by comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue. A recovery of more than 90 % was considered adequate to obtain required recovery.

Limit of detection (LOD) and limit of quantification (LOQ)

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.

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of CZ.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows:

$$\text{Matrix Factor} = \frac{\text{Peak response ratio in presence of extracted matrix (post extracted)}}{\text{Peak response ratio in aqueous standards}}$$

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Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV %) and %CV should be < 15%.

Calibration curve, precission and accuracy

The calibration curve was constructed using values ranging from 0.5 to 1500.0 ng/mL of CZ in rat plasma. Calibration curve was obtained by quadratic model with weighted $1/x^2$ regression analysis. The ratio of CZ / CZIS peak area was plotted against the ratio of CZ concentration in ng/mL. Calibration curve standard samples and quality control samples were prepared in replicates (n=6) for analysis. Precision and Accuracy for the back calculated concentrations of the calibration points, should be within d≤15 and ± 15% of their nominal values. However, for LLOQ, the Precision and Accuracy should be within d≤ 20 and ± 20%.

Stability (Freeze - thaw, Auto sampler, Bench top, Long term) of CZ in plasma

Low quality control and high quality control samples (n=6) were retrieved from a deepfreezer after three freeze-thaw cycles according to the clinical protocol. Samples were stored at -30 °C in three cycles of 24, 36 and 48 hr. In addition, the long-term stability of CZ in quality control samples was also evaluated by analysis after 65 days of storage at -30°C. Autosampler stability was studied following 73 hr storage period in the autosampler tray with control concentrations. Bench top stability was studied for 26 hr period with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The Precision and Accuracy for the stability samples must be d≤ 15 and ± 15 % respectively of their nominal concentrations.

Sample collection and statistical analysis

The validated method has been successfully used to analyze cilostazole concentrations in rat plasma. The study was conducted according to current GCP guidelines. Before conducting the study it was also approved by an authorized animal ethics committee. There were a total of 11 blood collection time points including the predose sample. The blood samples were collected in separate vacutainers containing K_2EDTA as anticoagulant. The plasma from these samples was separated by centrifugation at 3000 rpm within the range of 2–8 °C. The plasma samples thus obtained were stored at –30 °C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and 90% confidence interval was computed using SAS® software version 9.2.

RESULTS AND DISCUSSION

Method development

LC-MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The goal of this work is to develop and validate a simple, highly sensitive, rapid, rugged and reproducible assay method for the quantitative determination of CZ from rat plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, usage of different columns, different extraction methods such as solid phase, Precipitation, Liquid-liquid extraction methods were optimized through several trials to achieve the best resolution and increase the signal of CZ and CZIS. The MS optimization was performed by direct infusion of solutions of both CZ and CZIS into the ESI source of the mass spectrometer. The critical parameters in the ESI source include the needle (ESI) voltage, Capillary voltage, source temperature and other parameters such as nebulizer gas, heater gas and desolvation gases were optimized to obtain a better spray shape, resulting in better ionization of the protonated ionic CZ and CZIS molecules (Figure 2a-d). Product ion spectrum for CZ and CZIS yielded high-abundance fragment ions of *m/z* 124.9 and *m/z* 125.1 respectively (Figure. 2b and 2d). After mass spectrometer parameters optimized, chromatographic conditions such as mobile phase optimization, column optimization, extraction method optimization was performed to obtain a fast and selective LC method. A good separation and elution were achieved using 0.1% formic acid: acetonitrile (40:60v/v) as the mobile phase, at a flow-rate of 0.6 mL/min and injection volume of 10 µL. Princeton Spher-100 C18, 50 × 4.6 mm, 5 µm column and Liquid liquid extraction method was optimized for the best chromatography.(Figure. 4)

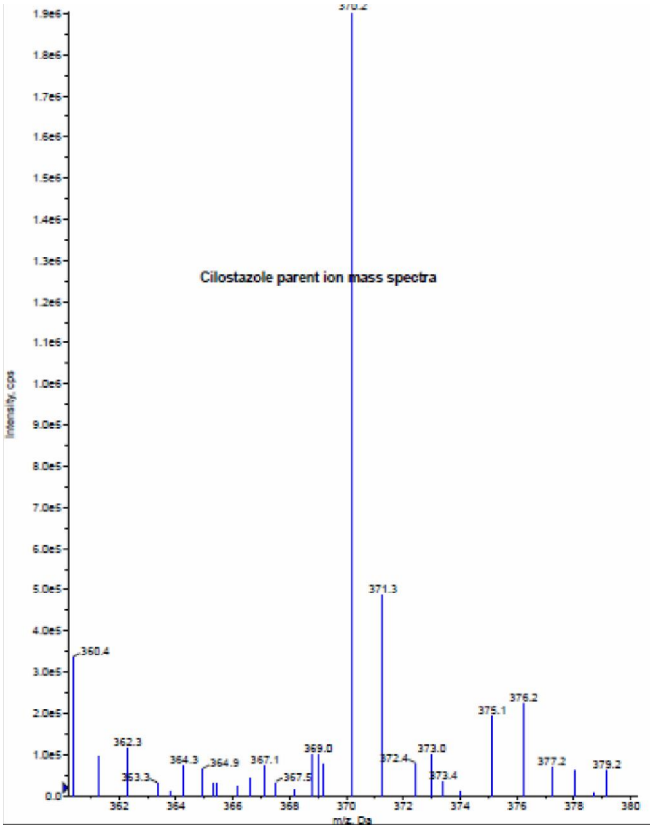

Figure 2a : Mass spectrum of Cilostazole Parent ion.

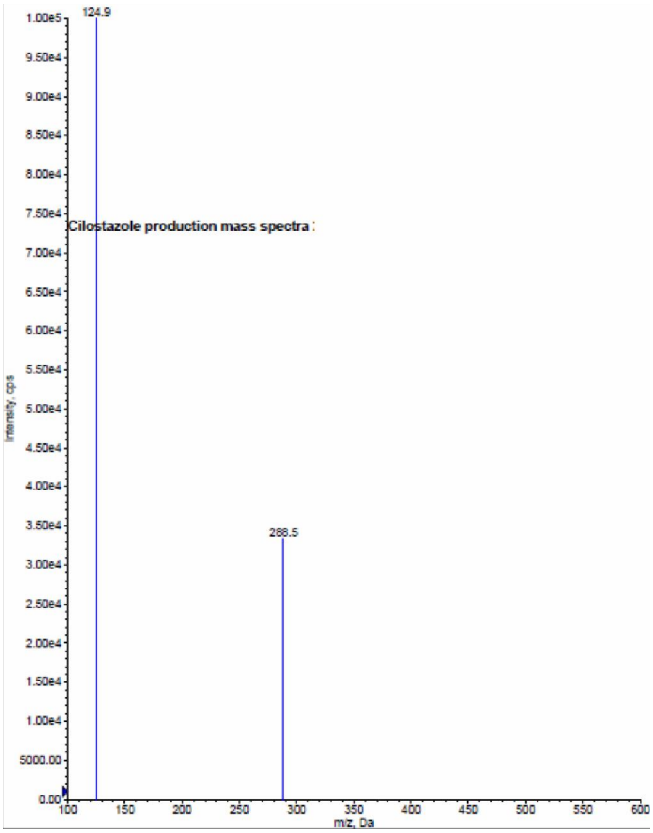

Figure 2b : Mass spectrum of Cilostazole Product ion.

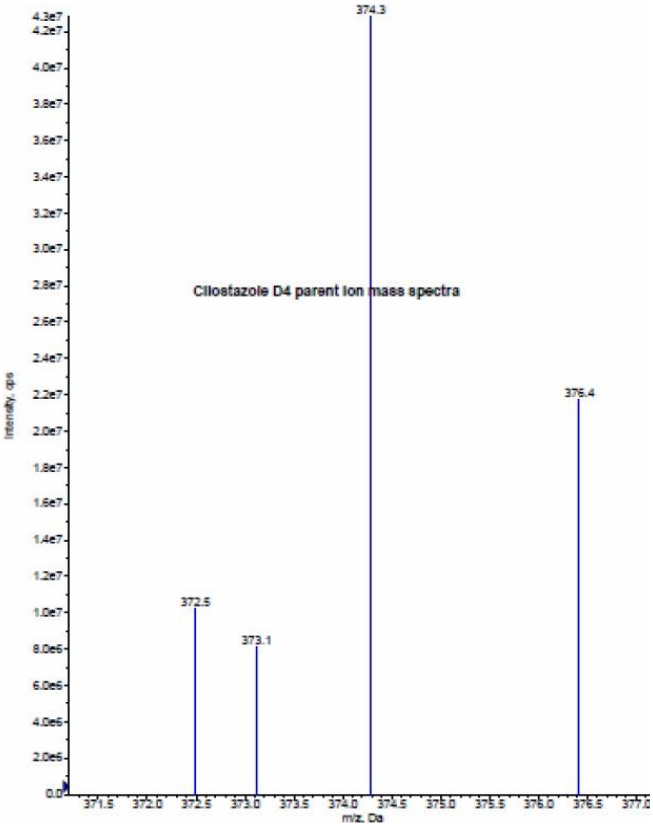

Figure 2c : Mass spectrum of Cilostazole D4 Parent ion.

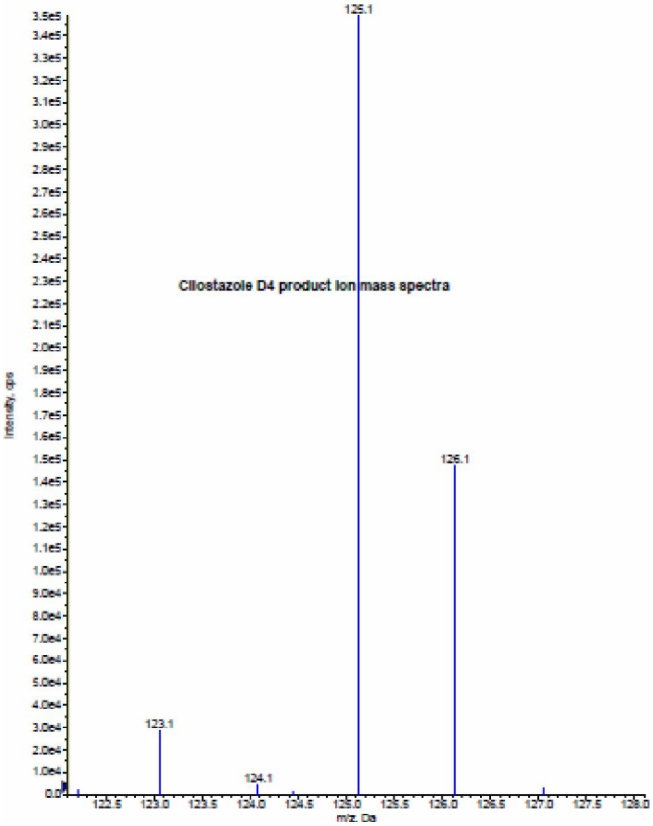

Figure 2d : Mass spectrum of Cilostazole D4 Product ion

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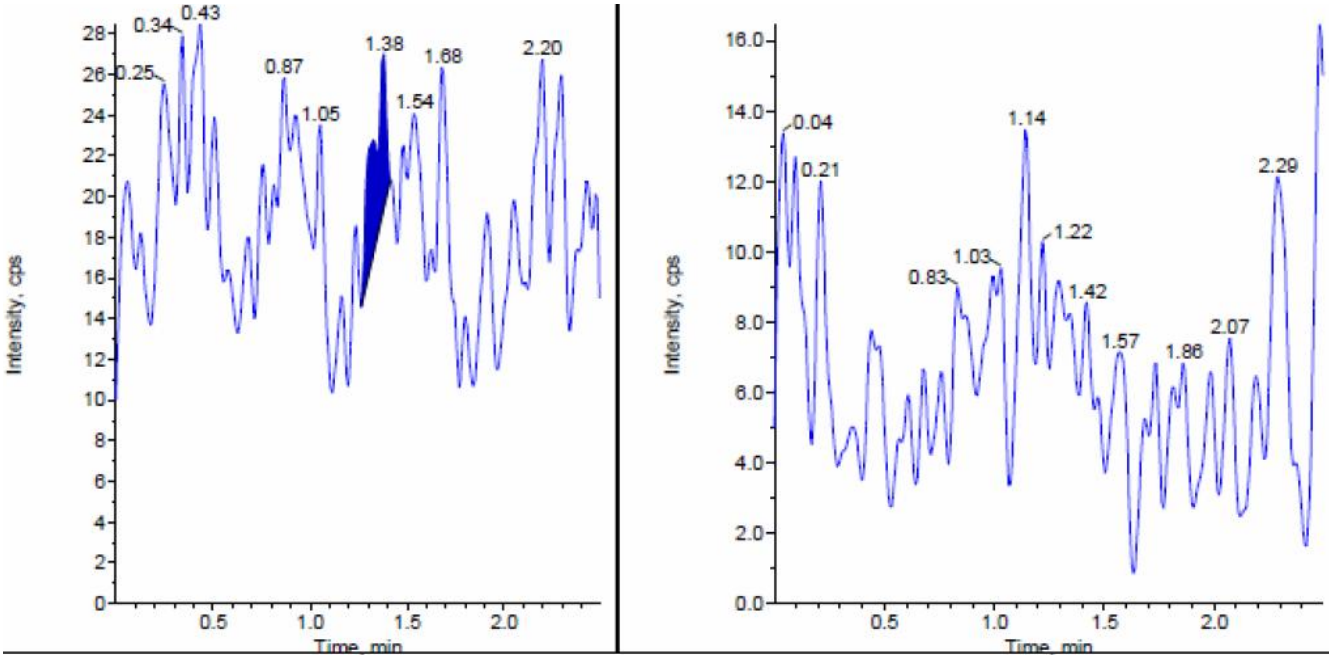

Figure 3 : Blank plasma chromatogram of Cilostazole and Cilostazole D4 in Rat plasma.

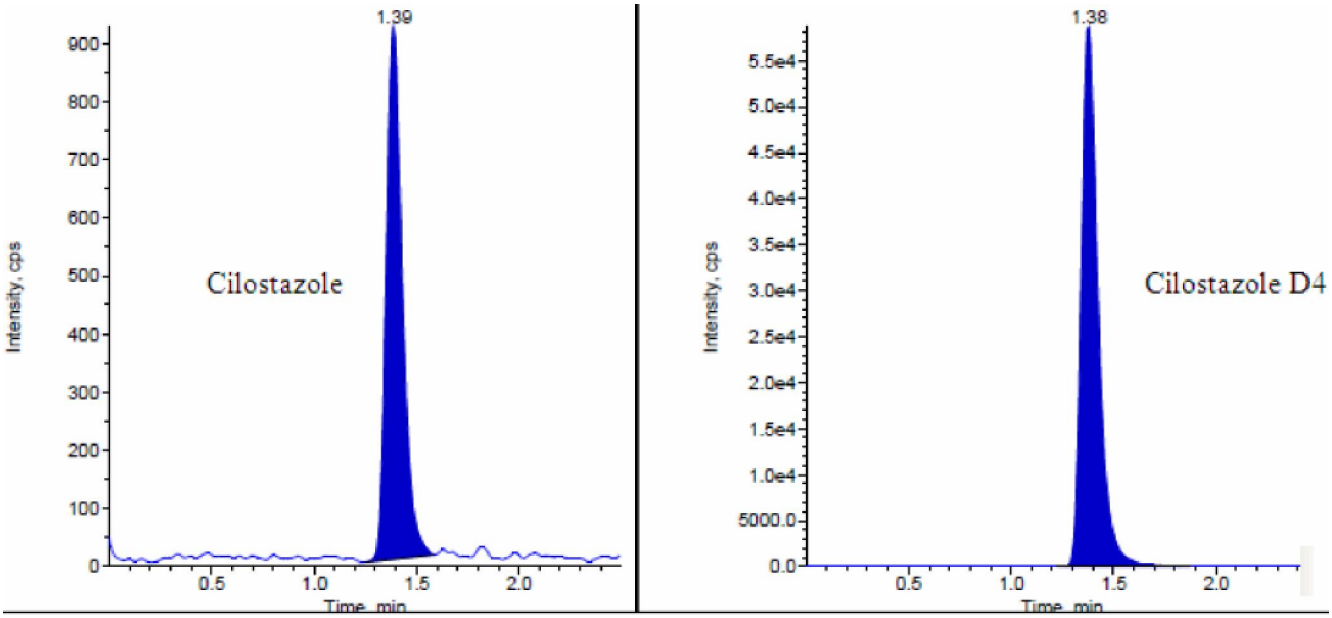

Figure 4 : LLOQ chromatogram of Cilostazole and Cilostazole D4 in Rat plasma.

Method validation

The developed method was validated over a linear concentration range 0.5-1550.0 ng/ml. The validation parameters include Selectivity and Specificity, Limit of Detection (LOD) and Quantification (LOQ), Matrix effect, Precision and Accuracy, Recovery, Stability (Freeze - thaw, Auto sampler, Bench top, Long term) was evaluated under validation section.

Selectivity and specificity

The analysis of CZ and CZIS using MRM (Multiple reaction monitoring) function was highly selective with no interfering compounds (Figure 3). Chromatograms obtained from plasma spiked with CZ (0.5 ng/mL) and CZIS (100.0 ng/mL) are shown in Figure 4.

Limit of detection (LOD) and quantification (LOQ)

The limit of detection was used to determine the instrument detection levels for CZ even at low concentrations. 10 µL of a 50.0 pg/mL solution was in-

jected and estimated LOD was 0.5 pg with S/N values e" 3-5.

The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 0.5 ng/ml.

Matrix effect

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor is 2.66 for Cilostazole. There was no ion- suppression and ion-Enhancement effect observed due to IS and analyte at respective retention time.

Precision and accuracy

Calibration curves were plotted as the peak area ratio (CZ/CZIS) versus (CZ) concentration. Precision and Accuracy of Calibration curve standards, Quality control standards represented in TABLE 1 and TABLE 2.

Stability (Freeze - thaw, Auto sampler, Bench top, Long term)

Quantification of the CZ in plasma subjected to three freeze-thaw cycles (-30°C to room temperature), Autosampler, Room temperature (Benchtop), Long term stability details were shown in TABLE 3.

Recovery

The recovery following the sample preparation using Liquid-liquid extraction with Methyl tertiary butyle ether was calculated by comparing the peak area of CZ in plasma samples with the peak area of solvent samples and was estimated at control levels of CZ. The recovery of CZ was determined at three different concentrations 1.5, 750.0 and 1000.0 ng/mL were found as 94.03, 95.01 and 94.03% respectively. The overall average recovery of CZ and CZIS were found to be 94.36 and 93.86% respectively.

Application to biological samples

The validated method has been successfully applied to quantify CZ concentrations in to a single dose (1.8mg/

TABLE 1 : Calibration curve details

Spiked plasma concentration (ng/mL)	Concentration measured(mean) (ng/mL)	SD	(%) CV (*n* = 5)	Accuracy %
0.5	0.50	0.49	0.01	1.10
1.0	1.00	1.10	0.02	1.70
5.0	5.00	5.20	0.12	2.30
10.0	10.00	9.90	0.28	2.80
75.0	75.00	73.70	3.02	4.10
150.0	150.00	148.80	3.87	2.60
300.0	300.00	289.50	9.84	3.40
600.0	600.00	597.40	43.61	7.30
900.0	900.00	912.20	25.54	2.80
1200.0	1200.00	1184.30	49.74	4.20
1500.0	1500.00	1489.60	47.67	3.20

TABLE 2 : Precision and accuracy (analysis with spiked plasma samples at four different concentrations)

Spiked plasma concen-tration (ng/mL)	Within-run			Between-run		
	Concentration measured (*n*=6) (ng/mL) (mean ± S.D)	(%) CV	% Accuracy	Concentration measured (*n*=30) (ng/mL) (Mean ±S.D.)	(%) CV	% Accuracy
0.5	0.49±0.00	1.00	98.00	0.46±0.01	1.25	92.00
1.5	1.46±0.02	1.46	97.33	1.45±0.04	2.45	96.67
750.0	751.20±20.13	2.68	100.16	745.67±27.07	3.63	99.42
1000.0	989.90±36.92	3.73	98.99	978.40±43.54	4.45	97.84

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Spiked plasma concentration (ng/ mL)	Room Temperature stability 26.0 h		Autosampler stability 73 h		Long term stability 65 days		Freeze and thaw stability Cycle 3 (48 h)	
	Concentration measured (n=6) (ng/ mL) (mean ±S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	% CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	%CV (*n*=6)
1.5	1.47±0.03	2.22	1.43±0.05	3.56	1.42±0.04	2.97	1.48±0.03	2.11
1000.0	985.26±36.16	3.67	956.86±41.72	4.36	946.78±36.55	3.86	989.65±28.90	2.92

200g) in rats. Male Sprague-Dawley rats were obtained from Bioneeds, Bangalore. After i. v administration of drug via left femoral vein 0.2 ml of blood samples for analytical determinations were collected via the right femoral vein at specific time intervals for 48 h. Plasma samples were stored at "30 æ%C until analysis. The study was carried out after approval from an independent animal ethics committee. The pharmacokinetic parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC0-48 (area under the plasma concentration–time curve measured 48 hours, using the trapezoidal rule), *T*max (time to observe maximum drug concentration), Kel (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression) and *T*1/2 (terminal half-life as determined by quotient 0.693/Kel). Pharmacokinetic details were shown in TABLE 4. The mean concentration versus time profile of CZ in rat plasma is shown in Figure5.

TABLE 4 : Mean Pharmacokinetic Parameters of Cilostazole in Rat plasma after intravenous administration of 1.8mg/ 200g male rat

Pharmacokinetic Parameter	values
$AUC_{0\text{-}t}$ (ng · h/mL)	9257.22
Cmax (ng/ mL)	839.65
$AUC_{0\text{-}?}$ (ng · h/mL)	9312.16
Kel	0.10957
Tmax (h)	1
$t_{1/2}$	6.32
ct	6.02
Ct/kel	54.94

$AUC_{0-\infty}$: area under the curve extrapolated to infinity; AUC_{0-t}: area under the curve up to the last sampling time; Cmax: the maximum plasma concentration; Tmax: the time to reach peak concentration; Kel: the apparent elimination rate constant.

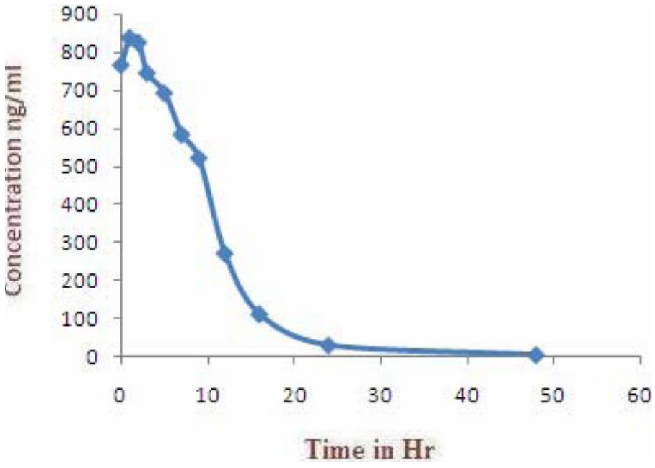

Figure 5 : Mean plasma concentrations vs. time graph of Cilostazole after intravenous administration of 1.8 mg/200g in male rat

ABBREVIATIONS

1 Liquid Chromatography-Electro Spray Ionization-Tandem Mass Spectrometry (LC–ESI–MS/MS)
2 Multiple Reaction Monitoring (MRM)
3 Cilostazole (CZ), Cilostazole d4(CZIS)
4 Lower limit of Quantification (LLOQ)
5 Limit of Quantification (LOQ), Low Quality Con trol (LQC)
6 Medium Quality Control (MQC)
7 High Quality Control (HQC)
8 Internal Standard (IS)
9 Collisionally Activated Dissociation (CAD)

CONCLUSION

The method described in this manuscript has been developed and validated over the concentration range of 0.5 - 1500.0 ng/mL in rat plasma. The selectivity,

sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. The simplicity of the method, and using rapid liquid–liquid extraction and sample turnover rate of 2.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of Cilostazole. The validated method was successfully applied in Pharmacokinetic study of rat plasma by intravenous administration of 9 mg/1 kg in 6 healthy male Wistor rats.

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