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ORIGINAL ARTICLE

Determination of cilostazol and its active metabolite 3,4-dehydro cilostazol from small plasma volume by UPLC–MS/MS

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KEYWORDS

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Sensitive;
High throughput

Abstract A simple, rapid and sensitive ultra performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) method has been developed for the simultaneous determination of cilostazol and its pharmacologically active metabolite 3,4-dehydro cilostazol in human plasma using deuterated analogs as internal standards (ISs). Plasma samples were prepared using solid phase extraction and chromatographic separation was performed on UPLC BEH C_{18} (50 mm × 2.1 mm, 1.7 μm) column. The method was established over a concentration range of 0.5–1000 ng/mL for cilostazol and 0.5–500 ng/mL for 3,4-dehydro cilostazol. Intra- and inter-batch precision (% CV) and accuracy for the analytes were found within 0.93–1.88 and 98.8–101.7% for cilostazol and 0.91–2.79 and 98.0–102.7% for the metabolite respectively. The assay recovery was within 95–97% for both the analytes and internal standards. The method was successfully applied to support a bioequivalence study of 100 mg cilostazol in 30 healthy subjects.

1. Introduction

Peripheral arterial disease (PAD) is a common manifestation of systemic atherosclerosis in which the blood vessels located outside the heart and brain are blocked or restricted. Intermittent claudication (IC) is one of the early symptoms of PAD. IC is characterized by stimulation of pain in leg muscles while walking relatively short distances and relieve of pain on rest. Cilostazol (CIL), a selective antagonist of phosphodiesterase (PDE) 3, was approved by US FDA in 1999 for the treatment of IC [1]. The efficacy of CIL for IC is mainly attributed to its vasodilatory actions and antiplatelet activity. Additionally, it shows antithrombotic effect, inhibits vascular smooth muscle proliferation, increases high-density lipoprotein and reduces

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serum glycerides. Other beneficial effects of CIL include endothelial cell activation, neuroprotective activity and scavenging free radicals [2]. CIL is an exceptional antiplatelet that shows minimal bleeding risk, and unlike other PDE3 inhibitors, it can be used in patients with cardiac comorbidities due to favorable effects on adenosine uptake and plasma lipid profile [3]. Most common adverse effects reported on the use of CIL include headache, diarrhea and palpitations. However, the symptoms are mild to moderate in severity and rarely require discontinuation of the drug [4]. CIL is rapidly absorbed after oral administration with a half life of 11–13 h, with peak plasma concentration attained at 2–4 h after administration. CIL is highly protein bound (95–98%) and is extensively metabolized in the liver via the cytochrome P450 enzymes, with metabolites largely excreted in urine [4,5]. CIL has been reported to metabolize to about 11 metabolites, among which 3,4-dehydro cilostazol (DCIL) is the major pharmacological active metabolite [6].

Simultaneous determination of CIL and its active metabolites is clinically important, especially DCIL, which is pharmacologically more potent (five times) than the parent drug [4]. Several methods were reported for the determination of CIL in biological fluids. The first method was reported for the determination of CIL as a single analyte in human plasma by reversed-phase HPLC [7]. Since then CIL was determined in the presence of its metabolites in human plasma [8–10], urine [11] and in liver microsomal incubation mixture [12] using either HPLC [9,11,12] or LC-MS/MS [8,10]. Yeon et al. [13] presented an automated microbore HPLC method with an on-line column switching system to determine CIL in human plasma. Varanasi et al. [14] developed and validated an LC-MS/MS method for simultaneous quantification of nateglinide, CIL, and DCIL in Wistar rat plasma. CIL was also determined by luminescence spectroscopy using Tb^{+3} as optical sensor in human serum and urine samples [15].

To the best of our knowledge, there are no reports on the simultaneous quantification of CIL and DCIL by UPLC−MS/MS. Thus, in the present work, a highly sensitive, rugged and rapid UPLC−MS/MS method was developed and fully validated as per the US FDA guidelines for the simultaneous estimation of CIL and DCIL in human plasma using deuterated internal standards. The method offered small turnaround time for analysis (1.2 min) and high sensitivity (0.5 ng/mL) for both the analytes and utilized only 100 µL human plasma for sample processing using solid phase extraction (SPE). The method was free from endogenous matrix interference and was successfully applied to a bioequivalence study with 100 mg cilostazol tablets in healthy subjects. The reproducibility in the measurement of subject samples was demonstrated by reanalysis of 125 incurred samples.

2. Experimental

2.1. Chemicals and materials

Reference standard of CIL (99.4%), CIL-d11 (99.3%), 3,4-dehydro cilostazol (DCIL, 99.5%) and DCIL-d11 (99.1%) were procured from Clearsynth Labs Pvt. Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). BioUltra grade ammonium formate and LC-MS grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). LiChroSep DVB-HL (30 mg, 1 cc) solid phase extraction cartridges were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Deionized water was obtained from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and was stored at −70 °C until use.

2.2. Chromatographic and mass detection conditions

Waters Acquity UPLC BEH C_{18} (50 mm × 2.1 mm, 1.7 µm) column maintained at 30 °C was used for chromatographic separation of the analytes. Elution of analytes and ISs was carried out using a mobile phase consisting of acetonitrile and 2.0 mM ammonium formate, pH 5.0 adjusted with 0.1% formic acid (80:20, *v/v*), delivered at a flow rate of 0.35 mL/min. The pressure of the system was maintained at 4500 psi. Quantitative determination was performed on Waters Quattro Premier XE (USA) triple quadrupole mass spectrometer equipped with electro-spray ionization (ESI) in the positive ionization mode. The main working parameters of the mass spectrometer are presented in Supplementary Table 1. MassLynx software version 4.1 was used to control all parameters of UPLC and MS.

2.3. Calibration standards and quality control samples

The standard stock solutions of CIL (400 µg/mL) and DCIL (200 µg/mL) were prepared by dissolving their requisite amounts in methanol. Further, working solutions were prepared using intermediate solutions of 200 and 20.0 µg/mL for CIL, 100 and 20.0 µg/mL for DCIL in methanol:water (50:50, v/v), respectively. The details of calibration standards (CSs) and quality control samples concentrations prepared by spiking of blank plasma with working solutions are given in Table 1. Separate stock solutions of the internal standards (100/50.0 µg/mL for CIL-d11/DCIL-d11) were prepared by dissolving accurately known amounts of ISs in methanol. Their working solutions were prepared from their stock solutions in methanol:water (50:50, v/v) at 800/400 ng/mL concentration for CIL-d11/DCIL-d11. Standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at −70 °C until use.

2.4. Extraction procedure

To an aliquot of 100 µL plasma sample, 25 µL of internal standard was added and vortexed for 10 s. Further, 100 µL of water was added and vortex mixed for another 30 s. The samples were centrifuged at 14,000*g* for 5 min at 10 °C and loaded on LiChroSep DVB-HL (30 mg, 1 cc) cartridges, pre-conditioned with 1.0 mL methanol followed by 1.0 mL of water. The samples were washed with 1.0 mL of 10% (v/v) methanol, followed by 1.0 mL of water. Thereafter, the cartridges were dried for 1 min under nitrogen (1.72×10^5 Pa) at 2.4 L/min flow rate. Both the analytes and ISs were eluted using 400 µL of acetonitrile into pre-labeled vials, followed by evaporation to dryness. The dried residue was reconstituted with 100 µL of mobile phase, briefly vortexed for 15 s and 10 µL was used for injection in the chromatographic system using an autosampler.

2.5. Validation procedures

Selectivity of the method was assessed for potential matrix interferences in 10 batches of blank human plasma by extraction and inspection of the resulting chromatograms for interfering peaks. The batches comprised 6 normal lots of K_3EDTA, 2 haemolysed and 2

Table 1 Calibration standards, quality control samples and assay performance of the proposed method.

Proposed method	Parameters	Cilostazol	3,4-dehydro cilostazol
Linearity assessment (calibration standards and quality control samples)	Linearity range (ng/mL)	0.5–1000	0.5–500
	Calibration standards (ng/mL)	0.50, 1.00, 3.00, 10.0, 20.0, 40.0, 100, 200, 500 and 1000	0.50, 1.00, 5.00, 20.0, 50.0, 100, 200, 300, 400 and 500
	Quality control samples (ng/mL)	0.50, 1.50, 150, 400 and 800	0.50, 1.50, 80.0, 200 and 420
	Weighting factor	$1/x^2$	$1/x^2$
	Mean regression line ($y=mx+c$)	$y=(0.002521\pm0.000019)\ x+(0.000032\pm0.000005)$	$y=(0.004966\pm0.000093)\ x+(0.000087\pm0.000038)$
	Correlation coefficient (r^2)	0.9998	0.9994
	Precision (% CV)	0.49–1.82	1.25–2.87
	Accuracy (%)	98.7–101.3	97.8–101.8
	LLOQ and LOD (ng/mL; *S/N* ratio)	0.50; ≥ 35 and 0.17; ≥ 13	0.50; ≥ 30 and 0.17; ≥ 10
Assay performance	System suitability[a]:		
	Precision (% CV)	0.35/ 0.84 for retention time/area response	0.27/ 0.71 for retention time/area response
	System performance[b]:		
	S/N ratio at LLOQ	≥ 35	≥ 25
	Autosampler carry-over[c]:		
	Blank plasma area response	≤ 9.74 ($\leq 1.33\%$ of LLOQ response)	≤ 7.64 ($\leq 1.41\%$ of LLOQ response)
	Method ruggedness[d]:		
	Precision (% CV)	0.75–1.19	0.68–1.83
	Accuracy (%)	Within 98.6–101.7	Within 98.2–102.1
	Dilution reliability[e]:		
	Precision (% CV)	0.65–0.96	0.72–1.13
	Accuracy (%)	Within 99.1–100.8	Within 98.8–100.7

[a]Aqueous standard mixture of analytes [at upper limit of quantitation (ULOQ)] and ISs injected as six consecutive injections at the start of each batch.

[b]One extracted blank (without analytes and ISs) and one extracted lower limit of quantitation (LLOQ) sample with ISs at the beginning of each analytical batch.

[c]Samples injection sequence: extracted blank plasma → ULOQ → two extracted blank plasma samples → LLOQ → extracted blank plasma.

[d]One QC batch analyzed on two BEH C_{18} columns with different batch numbers, while the second batch was analyzed by different analysts.

[e]Blank human plasma spiked with 1/5th and 1/10th dilution of the stock solution prepared at 2000/1000 ng/mL for cilostazol/3,4-dehydro cilostazol.

lipemic plasma. Interference of commonly used medications by human volunteers was also checked. This included paracetamol, chlorpheniramine maleate, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol:water (50:50, v/v). Further, working solutions were prepared in the mobile phase and 10 µL was injected to check for any possible interference at the retention time of analytes and ISs.

For linearity of the method, five calibration curves were plotted covering the range of 0.5–1000 ng/mL for CIL and 0.5–500 ng/mL for DCIL using least square regression and $1/x^2$ as a weighting factor. The area response ratio for analyte/IS obtained from multiple reaction monitoring was used for regression analysis. The acceptance criterion for a calibration curve was a correlation coefficient (r^2) ≥ 0.99 and the lowest standard on the calibration curve was accepted as the assay sensitivity expressed as LLOQ.

Intra-batch accuracy and precision were determined by analyzing six replicates of QC samples along with calibration curve standards on the same day, while the inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. The precision (% CV) at each concentration level from the nominal concentration was expected to be not greater than 15% and the accuracy to be within $\pm 15\%$ as per US FDA guidelines [16], except for the LLOQ where it could be 80–120% of the nominal concentration. Reinjection reproducibility was also checked by re-injecting one entire validation batch.

Ion suppression/enhancement effect was studied through post column analyte infusion experiment as described previously [17]. Briefly, standard solutions containing the analytes (at ULOQ level) and ISs were infused post column via a 'T' connector into the mobile phase. Suitable aliquots of extracted blank plasma (10 µL) were then injected into the column and MRM chromatograms were acquired for analytes and ISs to check any possible interference due to endogenous and exogenous plasma components.

The extraction recovery for the analytes and ISs was calculated by comparing the mean area response of samples ($n=6$) spiked before extraction to that of extracts with post-spiked samples (spiked after extraction) at four QC levels. Matrix effect, expressed as matrix factors (MFs), was assessed by comparing the mean area response of post-spiked samples with samples prepared in mobile phase. IS-normalized MFs (analyte/IS) were calculated to access the variability of the assay due to matrix effects. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibration curves prepared from eight plasma lots, which included haemolysed and lipemic plasma samples. To prove the absence of matrix interference, % CV should not be greater than 3–4% [18].

Stability tests were conducted for stock solutions of analytes and ISs for short term and long term stability at 25 and 5 °C, respectively. All

stability results for spiked plasma samples were evaluated by measuring the area response ratio (analyte/IS) of stability samples against freshly prepared comparison standards. QC samples at HQC and LQC levels were prepared to check for bench top, wet extract (autosampler), processed sample, dry extract, freeze–thaw (−20 and −70 °C) and long term (−20 and −70 °C) stabilities. The acceptance criterion was ±10.0% deviation (from the nominal value) for stock solutions and ±15.0% deviation for all other storage conditions.

2.6. *Application to a bioequivalence study*

The bioequivalence study was done with a test (100 mg cilostazol tablets from Aché Laboratórios Farmaceuticos, Brazil) and a reference formulation (Cebralat®, Libbs Pharmaceuticals, Ltd., Brazil) in 30 healthy male subjects with a washout period of 10 days between dosing. The inclusion criteria for subject selection were based on the age (18–40 years), mean weight of 63 kg, body mass index (between 18.5 and 30.0 kg/height^2), general physical examination and vital signs (blood pressure, pulse rate, respiration rate and oral temperature), electrocardiogram and laboratory tests like hematology, blood chemistry, urine examination and immunological tests. The exclusion criteria included allergic responses to CIL, volunteers with history of alcoholism, smoking and having a disease which may compromise the haemopoietic, gastrointestinal, renal, hepatic, cardiovascular, respiratory, central nervous systems, or any other body system as well as diabetes, psychosis. The study was conducted as per the International Conference on Harmonization and US FDA guidelines [19]. The subjects were informed about the objectives and possible risks involved in the study and a written consent was obtained. The subjects were orally administered a single dose of test and reference formulations with 240 mL of water. Blood samples were collected at 0.00 (predosing), 0.50, 1.00, 1.50, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.00, 4.50, 5.00, 6.00, 8.00, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 24.0, 36.0, 48.0 and 72.0 h after oral administration of the dose for test and reference formulations in labeled K_3EDTA-vacuettes. Plasma was separated by centrifugation and kept frozen at −70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. For the purpose of bioequivalence analysis, C_{max}, $AUC_{0–72\ h}$ and $AUC_{0–inf}$ were considered as primary variables. The pharmacokinetic parameters of CIL and DCIL were estimated by non-compartmental analysis using WinNonlin® software version 5.3 (Pharsight Corporation, Sunnyvale, CA, USA).

3. Results and discussion

3.1. *UPLC–MS/MS method development*

Although there are several methods available in the literature for the simultaneous determination of CIL and DCIL, they have a limitation either in terms of sensitivity [7,9,11–14], overall

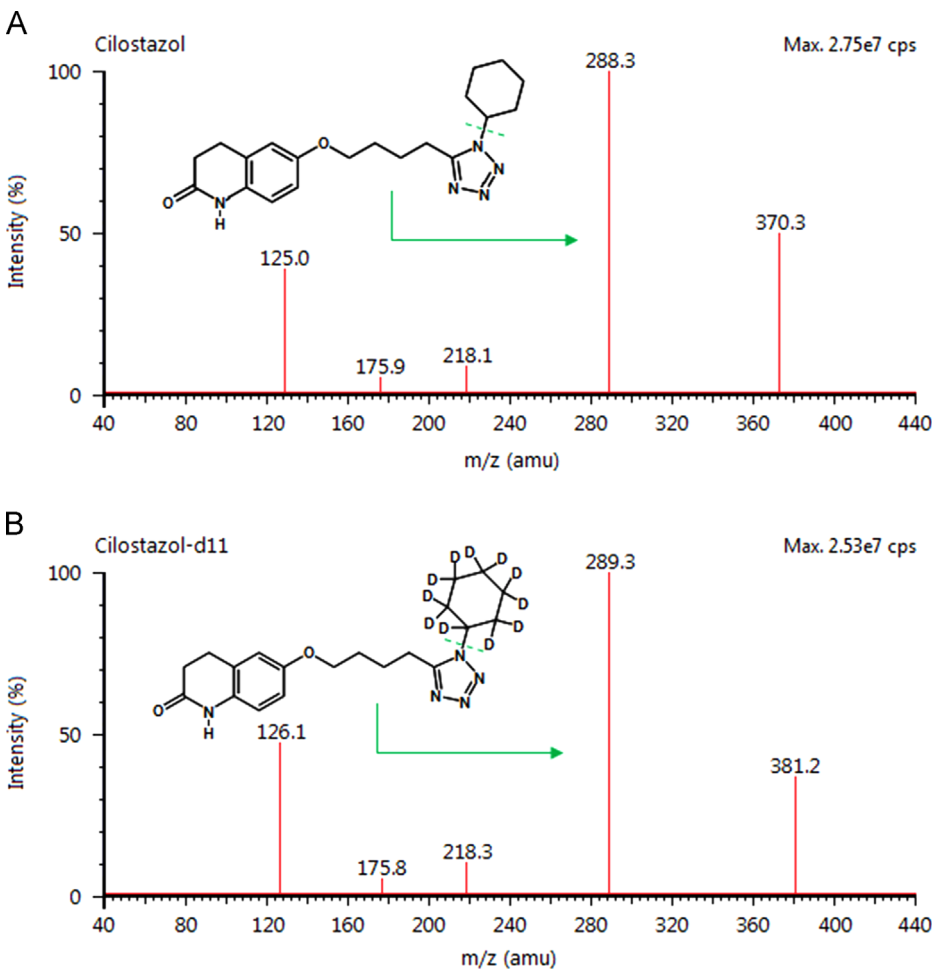

Fig. 1 Product ion mass spectra in the positive ionization mode for (A) cilostazol (*m/z* 370.3→288.3) and (B) cilostazol-d11, IS (*m/z* 381.2→288.3).

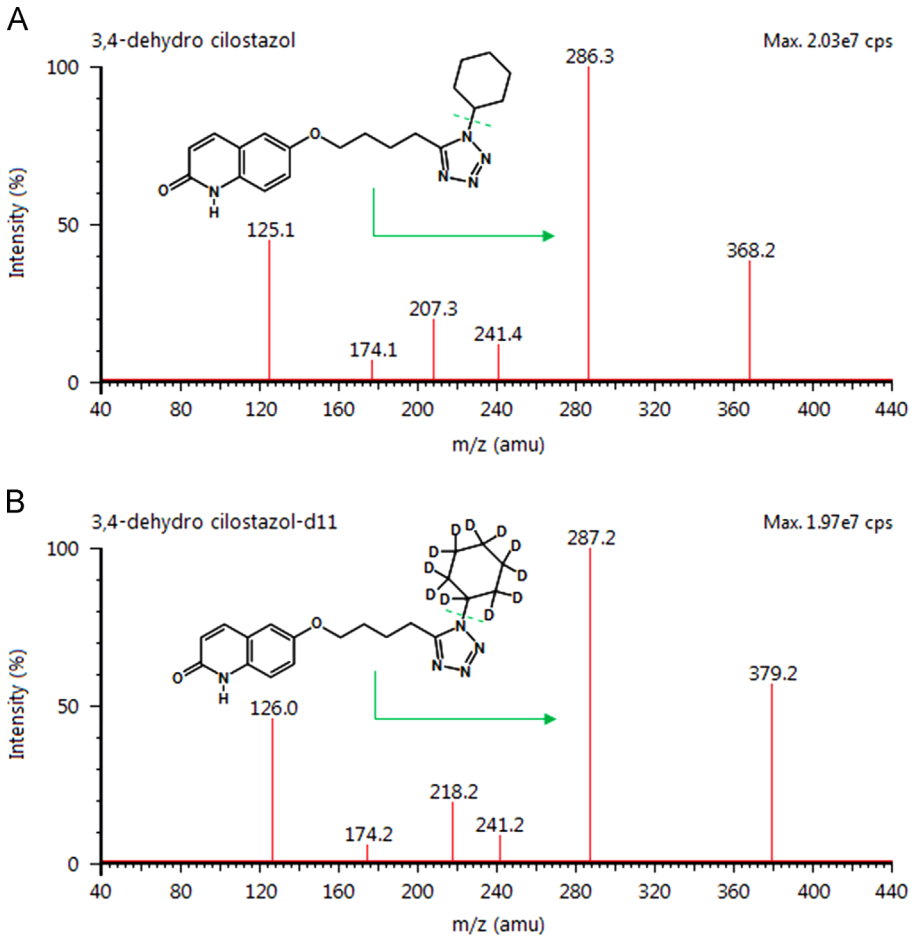

Fig. 2 Product ion mass spectra in the positive ionization mode for (A) 3,4-dehydro cilostazol (*m/z* 368.2→286.3) and (B) 3,4-dehydro cilostazol-d11, IS (*m/z* 379.2→286.2).

analysis time [7–9,11–13], plasma processing volume [7–10] or cumbersome extraction procedure [7–9]. Thus, the aim of the work was to develop a rugged UPLC−MS/MS method which offered combined advantage of sensitivity, selectivity, simplicity of extraction procedure and high throughput. Unlike all previous methods, deuterated internal standards were used, which could adequately compensate for any variability during extraction and analysis, thereby ensuring the accuracy of the generated data. UPLC could provide a superior alternative to HPLC, especially in reducing the analysis time when large numbers of samples were to be analyzed in a clinical setting. This technology was capable of achieving higher peak capacity, speed and sensitivity than conventional HPLC. In addition, solvent consumption could be considerably reduced compared to conventional 4.6 mm id columns [20]. Further, tandem mass spectrometric detection was expected to provide improved limits of detection and selectivity in the multiple reaction monitoring (MRM) mode.

As both the analytes and ISs possess easily ionizable amino groups, electrospray ionization (ESI) in the positive ion mode was used for MRM analyses. The tandem mass spectrometry parameters were optimized to maximize the response for the analytes and ISs (Supplementary Table 1). The Q1 MS full scan spectra showed consistent and predominant protonated $[M+H]^+$ precursor ions at *m/z* 370.3, 381.2, 368.2 and 379.2 for CIL, CIL-d11, DCIL and DCIL-d11 respectively. The product ion mass spectra under the optimized conditions for the analytes and ISs are presented in Figs. 1 and 2. The most stable and consistent fragments ions for CIL, CIL-d11, DCIL and DCIL-d11 were observed at *m/z* 288.3, 288.3, 286.3 and 286.2, respectively, as a result of selective loss of cyclohexyl (deuterated for ISs) moiety from the protonated precursor ions.

Several reports recommended combination of two extraction procedures for selective extraction of CIL and its metabolites from endogenous substances for improved recovery from human plasma [7–9]. Out of the two procedures, one is liquid–liquid extraction (LLE) as both the analytes are lipophilic in nature with Log *D* values of 3.30 and 3.38 for CIL and DCIL respectively. Akiyama et al. [7] proposed combination of protein precipitation (PP) and LLE for CIL extraction from plasma. Similarly, two other reports applied LLE first followed by SPE to obtain clear extracts for CIL and its metabolites [8,9]. Both these methods required multiple steps to process the aqueous as well as organic portions for improved recovery and precision. Nirogi et al. [10] proposed a simplified approach with LLE using a mixture of diethyl ether and dichloromethane; however, the recovery was comparatively less (69–71%). Yeon et al. [13] employed filtration of plasma samples using a low protein binding membrane syringe filter after diluting the samples with water. The recovery obtained for CIL by this approach was quantitative; nevertheless, it required a pre-column for on-line sample preparation. In the present study, several trials were carried out with all three extraction techniques namely PP, LLE and SPE. PP was tried out using methanol and acetonitrile as protein precipitants; however, the recovery was low with high variability (40–65%) at LLOQ and LQC levels for both the analytes. Further, LLE was tested

Table 2 Extraction recovery and matrix factor for cilostazol and 3,4-dehydro cilostazol.

Analyte	QC level	Area response ($n=6$)			Extraction recovery (%) (B/A)	Matrix factor		
		A	B	C		Analyte (A/C)	IS	IS-normalized
Cilostazol	LQC	2277	2172	2219	95.4 (95.8)[a]	1.026	1.015	1.011
	MQC-2	221,645	214,012	218,648	96.6 (95.1)[a]	1.014	0.988	1.026
	MQC-1	589,972	570,485	585,478	96.7 (96.5)[a]	1.008	0.995	1.012
	HQC	1,187,945	1,142,988	1,180,245	96.2 (96.4)[a]	1.007	1.002	1.005
3,4-dehydro cilostazol	LQC	1628	1553	1585	95.4 (95.8)[b]	1.027	1.019	1.008
	MQC-2	87,385	84,258	86,139	96.4 (96.3)[b]	1.014	0.995	1.019
	MQC-1	220,174	210,452	215,681	95.6 (95.1)[b]	1.021	1.017	1.004
	HQC	455,982	434,658	448,523	95.3 (95.2)[b]	1.017	1.007	1.010

A: mean area response of six replicates prepared by spiking in extracted blank plasma; B: mean area response of six replicates prepared by spiking before extraction; C: mean area response of six replicates prepared by spiking in mobile phase (neat samples); *n*: number of replicates; IS: Internal standard; LQC: low quality control; MQC: medium quality control; HQC: high quality control.

[a]Values for internal standard, cilostazol-d11.

[b]Values for internal standard, 3,4-dehydro cilostazol-d11.

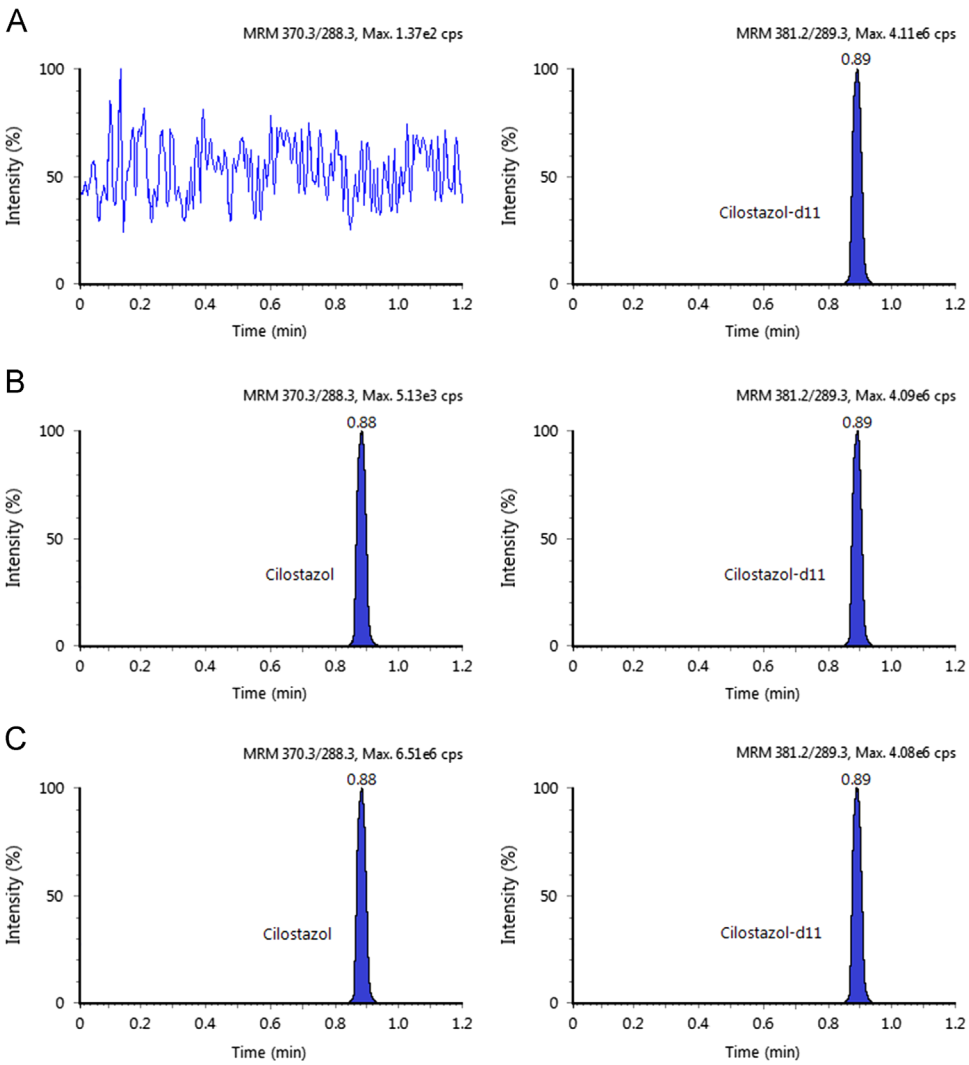

Fig. 3 Representative chromatograms of cilostazol (*m/z* 370.3→288.3) and cilostazol-d11 (*m/z* 381.2→288.3) in (A) blank plasma, (B) analytes at LLOQ and (C) real subject sample at C_{max} after administration of 100 mg dose of cilostazol tablet.

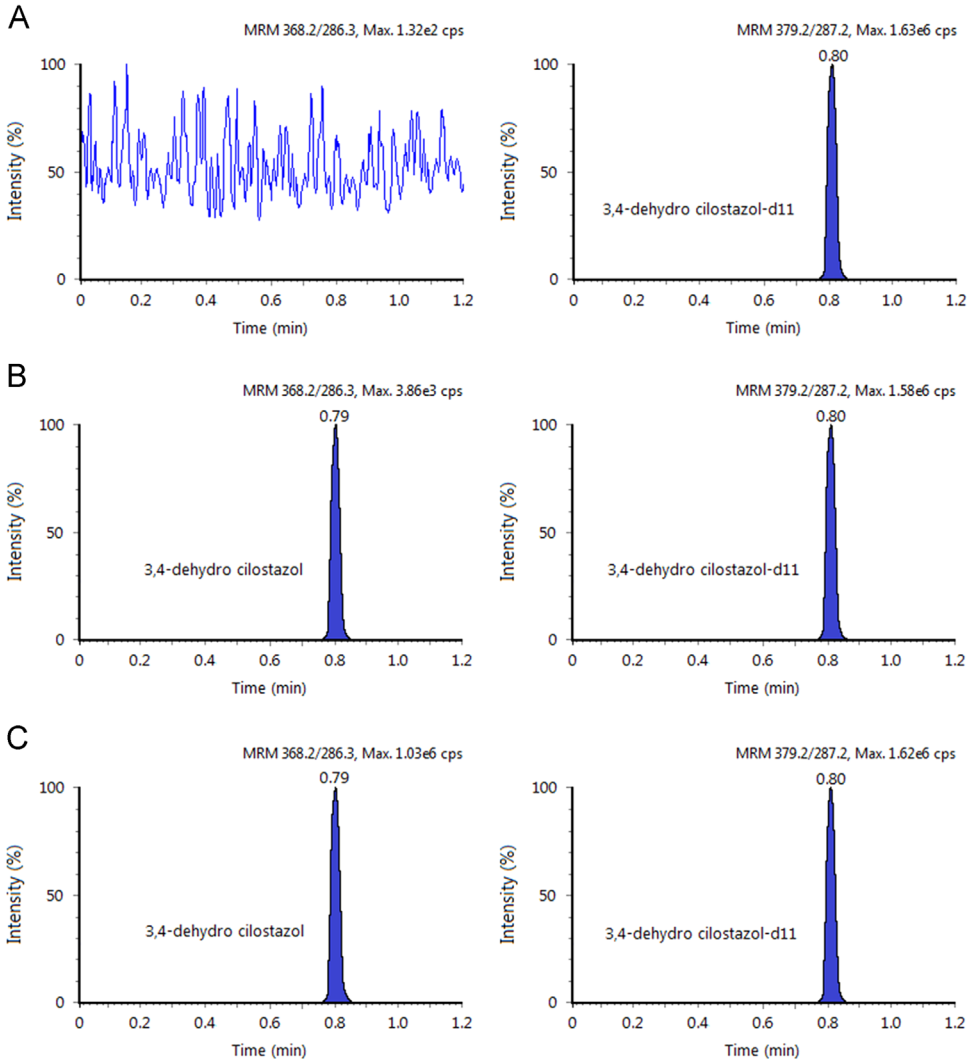

Fig. 4 Representative chromatograms of 3,4-dehydro cilostazol (*m/z* 368.2→286.3) and 3,4-dehydro cilostazol-d11 (*m/z* 379.2→286.2) in (A) blank plasma, (B) analytes at LLOQ and (C) real subject sample at C_{max} after administration of 100 mg dose of cilostazol tablet.

with different organic diluents like dichloromethane, diethyl ether, chloroform, *n*-hexane, TBME, alone and in combination under neutral and alkaline conditions as suggested in the reported methods [7–11,14]. The results showed poor recovery (55–65%) in all most all the solvents especially for LLOQ, LQC and MQC-2 samples. Thus, SPE was carried out on LiChroSep DVB-HL cartridges (30 mg, 1 cc) to overcome the problems encountered during PP and LLE. These cartridges contain reversed phase functionalized polymeric sorbent that gives adequate retention for basic, acidic and neutral compounds. The clear samples with high efficiency were obtained by SPE under the optimum conditions as shown in Table 2. Quantitative and precise recovery (95–97%) was obtained across all QC levels for both the analytes.

A majority of the methods used acetonitrile–ammonium acetate buffer as the mobile phase for chromatographic analysis of CIL and its metabolites under gradient program with a very long analysis time [8,9,11–13]. Tata et al. [12] used two HPLC columns in tandem for improved chromatographic efficiency and resolution to get quantitative recovery and overcome matrix interferences, while Yeon et al. [13] set up an on-line triple column switching system for removal of plasma proteins and sample enrichment. Both these methods took unduly long time for separation of analytes under gradient elution and thus might not be useful for high throughput analysis. Besides, both of these methods required significant modifications to existing HPLC systems. Thus for optimum separation of analytes, several chromatographic parameters were investigated on Acquity UPLC BEH C_{18} (50 mm × 2.1 mm, 1.7 µm) column, including the type of organic modifier, buffer, the concentration and pH of the buffer, and organic modifier:buffer ratio. Initial trials were conducted using acetonitrile/methanol as an organic modifier along with mobile phase additives like ammonium formate and ammonium acetate in the pH range of 2.0–5.0. Based on the primary outcomes, acetonitrile and ammonium formate were chosen for further optimization based on peak shape and adequate response. The sensitivity was significantly increased in presence of ammonium formate compared to ammonium acetate buffer with about 1.5 time higher peak areas of CIL and DCIL. Further, the organic modifier:buffer ratio and pH of the buffer were optimized for better resolution without compromising the response for the analytes. Acetonitrile content of 80% at a flow rate of 0.350 mL/min ensured adequate retention and peak resolution between the analytes. It was observed that the pH of the mobile phase influences both the chromatographic elution of the compounds and the formation of the $[M+H]^+$ molecular ions. As both the analytes and ISs are basic compounds, the use of slightly acidic solution

favors ionization of the analytes in ESI by protonation of their basic sites. A mobile phase pH of 5.0 ensured adequate response for the analytes with high sensitivity. CIL and DCIL were baseline resolved within 1.2 min with a resolution factor of 1.13, using acetonitrile–2.0 mM ammonium formate, pH 5.0 adjusted with 0.1% formic acid (80:20, v/v) as a mobile phase (Figs. 3 and 4). The capacity factors and number of theoretical plates were 1.51 and 1936 for CIL and 1.23 and 1560 for DCIL, respectively. The efficiency of sample cleanup and chromatography can be demonstrated by flat baseline, with negligible influence of endogenous and exogenous plasma components at the retention time of the analytes and ISs. To achieve the desired sensitivity with acceptable accuracy and precision, separate ISs were used for the drug and its active metabolite. Deuterated ISs used in the present work helped to overcome any possible matrix effects, errors in sample preparation and quantitative measurements for reliable results.

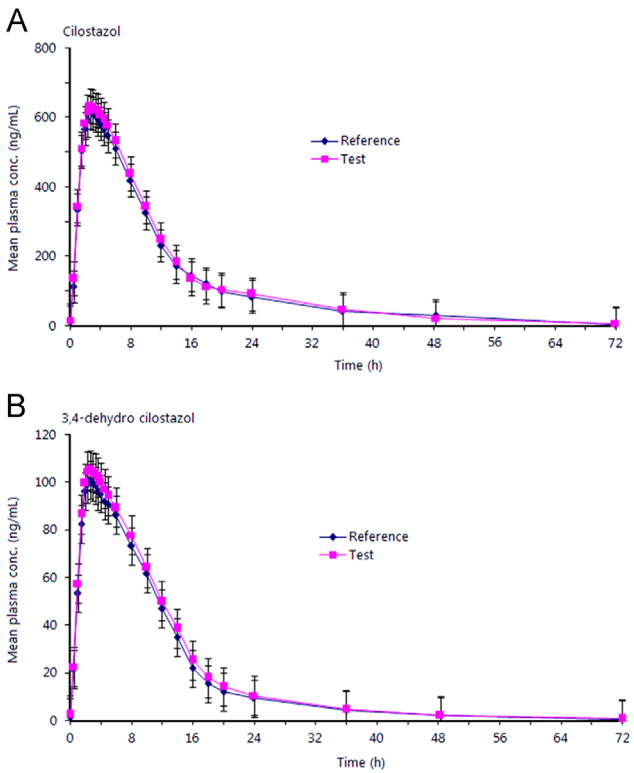

Fig. 5 Mean plasma concentration–time profile of (A) cilostazol and (B) 3,4-dehydro cilostazol after oral administration of test (100 mg cilostazol tablets from Aché Laboratórios Farmacêuticos, Brazil) and reference (Cebralat®, 100 mg of cilostazol tablets from Libbs Pharmaceuticals Ltd., Brazil) formulations to 30 healthy Indian subjects under fasting condition.

3.2. *Assay performance and selectivity*

System suitability, system performance, and carry-over data indicate acceptable assay performance of the method as shown in Table 1. Both the analytes showed good linearity over their specified range ($r^2 \geq 0.9994$). The reinjection reproducibility in the measurement of retention times for the analytes, expressed as % CV, was ≤ 0.81 for 100 injections on the same column. The limit of detection (LOD) and LLOQ of the method were 0.17 and 0.50 ng/mL for both the analytes at a signal-to-noise ratio of ≥ 10 and ≥ 30, respectively (Table 1).

The selectivity of the method can be seen from Figs. 3 and 4 in blank plasma spiked with ISs, analytes at LLOQ and subject sample at C_{max}, which show no direct interference due to endogenous components at the retention of the analytes and ISs. Additionally, none of the commonly used medications by human volunteers interfered at their respective retention times.

3.3. *Accuracy and precision, recovery and matrix factors*

The intra-batch precision (% CV) ranged from 0.91% to 1.99% and the accuracy was within 98.0–101.7% for both the analytes.

Table 3 Pharmacokinetic parameters (mean ± SD) and comparison of treatment ratios and 90% CIs of natural log (Ln)-transformed parameters following oral administration of 100 mg cilostazol tablet formulation in 30 healthy Indian subjects under fasting.

Analyte	Parameter	Test	Reference	Ratio (test/reference) (%)	90% CI (lower–upper)	Power	Intra subject variation (CV, %)
Cilostazol	C_{max} (ng/mL)	635.6 ± 86.2	623.3 ± 79.9	102.0	97.3–106.6	0.9996	5.38
	$AUC_{0-72\,h}$ (h ng/mL)	8532.2 ± 587.1	8567.2 ± 613.4	99.6	94.1–105.4	0.9994	6.74
	AUC_{0-inf} (h ng/mL)	8832.6 ± 714.3	8914.5 ± 749.4	99.1	92.8–105.5	0.9997	7.15
	T_{max} (h)	2.33 ± 0.30	2.52 ± 0.27	–	–	–	–
	$t_{1/2}$ (h)	11.40 ± 0.35	11.46 ± 0.28	–	–	–	–
	K_{el} (1/h)	0.061 ± 0.002	0.061 ± 0.001	–	–	–	–
	V/F (L)	157.3 ± 3.1	160.4 ± 4.8	–	–	–	–
3,4-dehydro cilostazol	C_{max} (ng/mL)	115.2 ± 13.8	109.9 ± 11.3	104.9	99.8–109.4	0.9993	4.86
	$AUC_{0-72\,h}$ (h ng/mL)	1553.8 ± 312.3	1579.1 ± 326.8	98.4	93.7–103.8	0.9995	5.69
	AUC_{0-inf} (h ng/mL)	1695.1 ± 331.8	1727.8 ± 354.2	98.1	94.0–103.2	0.9999	4.62
	T_{max} (h)	2.24 ± 0.37	2.47 ± 0.25	–	–	–	–
	$t_{1/2}$ (h)	11.26 ± 0.23	11.53 ± 0.44	–	–	–	–
	K_{el} (1/h)	0.062 ± 0.002	0.060 ± 0.003	–	–	–	–
	V/F (L)	868.1 ± 13.1	909.9 ± 16.7	–	–	–	–

CI: confidence interval; CV: coefficient of variation; C_{max}: maximum plasma concentration; $AUC_{0-72\,h}$: area under the plasma concentration–time curve from zero hour to 72 h; AUC_{0-inf}: area under the plasma concentration–time curve from zero hour to infinity; T_{max}: time point of maximum plasma concentration; $t_{1/2}$: half life of drug elimination during the terminal phase; K_{el}: elimination rate constant; V/F: volume of distribution; SD: standard deviation.

Likewise for inter-batch experiments, the precision varied from 0.99% to 2.79% and the accuracy was within 98.1–102.7% (Supplementary Table 2). The extraction recovery and IS-normalized MF for the analytes are presented in Table 2. The mean extraction recovery varied from 95.4% to 96.7% for CIL and 95.3% to 96.4% for DCIL at all QC levels. As presence of unmonitored, co-eluting compounds from the matrix can directly impact the overall reliability of a validated method, it is recommended to evaluate MFs to consider the matrix effect [21]. Additionally, it is required to check the matrix effect in lipemic and haemolysed plasma samples together with normal K_3EDTA plasma. The IS-normalized MFs using stable-isotope labeled IS should be close to unity due to similarities in the chemical properties and elution behavior of the analytes and ISs. The IS-normalized MFs ranged from 1.004 to 1.026 for both the analytes.

In addition, interferences due to endogenous plasma components were also assessed by plotting calibration curves for eight different batches of blank plasma lots. The coefficient of variation (% CV) of the slopes of calibration lines for relative matrix effect in eight different plasma lots was 1.79 and 1.46 for CIL and DCIL, respectively (Supplementary Table 3). Furthermore, the extracts obtained through SPE showed negligible matrix effect, which were analyzed by the post column analyte infusion method. The results confirmed the absence of signal suppression or enhancement at the retention time of the analytes and ISs (Supplementary Fig. 1).

3.4. *Stability, dilution reliability and method ruggedness*

Stock solutions kept for short-term and long-term stability as well as spiked plasma solutions showed no evidence of degradation under all studied conditions. No significant degradation was observed for both the analytes during sample storage and any of the processing steps during extraction. The detailed results for stability studies are presented in Supplementary Table 4. The precision values for method ruggedness on different BEH C_{18} (50 mm × 2.1 mm, 1.7 µm) columns and with different analysts were within 0.68–1.83%. The ability to dilute samples which could be above the upper limit of the calibration range was validated by analyzing six replicate samples containing 2000 ng/mL of CIL and

Table 4 Comparative assessment of chromatographic methods developed for cilostazol and its metabolite(s) in human plasma.

Sr. no.	Technique; linear range (ng/mL)	Extraction procedure; plasma volume (µL); internal standard; mean extraction recovery (%)	Column; mobile phase; run time (min); flow rate (mL/min)	Maximum on-column loading per injection volume (ng); organic solvent consumption (mL, approximate value)	Application; matrix effect study; ISR results (% change)	Ref.
1	HPLC; 25–2000	PP with ACN followed by LLE; 1000; OPC-13012; 74.1	µBondapak C_{18} RP (300 mm × 3.9 mm, 10 µm); ACN–water (42:58); 16.0; 1.7	800; 25	Pharmacokinetic study with 100 mg CIL in 12 healthy subjects; NA; NA	[7]
2[a]	HPLC; 20–1200 for all the analytes	LLE followed by SPE; 1000; OPC-3930 and OPC-13112; 99.6–104.9 for all the analytes	TSK-GEL ODS-80TM (150 mm × 4.6 mm, 5 µm); Gradient elution with ACN–100 mM acetate buffer (10:90 and 60:40); 75/55; 1.0	240; 43	NA; NA; NA	[8]
3[b]	LC-MS/MS; 5.0–1200 for all the analytes	LLE followed by SPE; 500; OPC-3930; 88.2–106.6 for all the analytes	Supercosil LC-18-D8 (150 mm × 4.6 mm, 5 µm); Gradient elution with ACN–acetate buffer (10:90 and 90:10); 17.5; 1.0	12; 28	NA; NA; NA	[9]
4[c]	LC-MS/MS; 5.0–2000 for CIL and 5–400 for DCIL	LLE; 500; mosapride; 71.5/69.2 for CIL/DCIL	Inertsil C_{18} (150 mm × 4.6 mm, 5 µm); ACN–5 mM acetate buffer (90:10); 2.5; 1.2	400/80 for CIL/DCIL; 7.0	Pharmacokinetic study with 100 mg CIL in 6 healthy subjects; NA; NA	[10]
5	HPLC; 25–2000	Dilution followed by filtration through protein binding membrane syringe filter; 240; NA; 98.9	Two Capcell Pak MF Ph-1 columns (20/35 mm × 4.0/2.0 mm, 5 µm) followed by one Capcell Pak C18 UG 120 (250 mm × 1.5 mm, 5 µm); ACN–water (10:90 and 40:60); 21; 1.0 and 0.150	480; 3.5	Pharmacokinetic study with 100 mg oral dose of CIL in 16 human subjects; NA; NA	[13]
6[c]	UPLC–MS/MS; 0.5–1000 for CIL and 0.5–500 for DCIL	SPE; 100; CIL-d11, DCIL-d11; 96.2/95.6 for CIL/DCIL	Waters Acquity BEH C_{18} column; 2 mM ammonium formate–ACN (20:80), pH 5.0; 1.2; 0.35	10/5 for CIL/DCIL; 2.5	Bioequivalence study with 100 mg CIL in 30 healthy subjects; Post-column infusion study; within ±12%	PS

ISR: incurred sample reanalysis; PP: protein precipitation; ACN: acetonitrile; CIL: cilostazol; LLE: liquid–liquid extraction; SPE: solid phase extraction; NA: not available; PS: present study.

[a]Cilostazol and its seven metabolites.

[b]Cilostazol with its three metabolites.

[c]Cilostazol with 3,4-dehydro cilostazol (DCIL).

1000 ng/mL of DCIL after five-/ten-fold dilution, respectively. The precision (% CV) values for dilution reliability were between 0.72 and 1.13 for both the dilutions (Table 1).

3.5. Application of the method in healthy subjects and incurred sample reanalysis results

Fig. 5 shows the plasma concentration vs. time profile for CIL and DCIL under fasting condition. The results showed that the newly developed analytical method had the required sensitivity to characterize the absorption, distribution and elimination phases of cilostazol following oral dosing. A similar study with 100 mg dose of CIL was reported in six healthy Indian subjects [10]. However, the values for pharmacokinetic parameters were not available for comparison. Table 3 summarizes the mean pharmacokinetic parameters after oral administration of 100 mg cilostazol tablet formulation in 30 subjects. The T_{max}, C_{max} and AUC values obtained for CIL in the present work were comparable with two previous reports [13,22]. Further, no statistically significant differences were found between the two formulations in any parameter. The ratios of mean log-transformed parameters (C_{max}, $AUC_{0-72\,h}$ and AUC_{0-inf}) and their 90% CIs were all within the defined bioequivalence range of 80–125% (Table 3). These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption.

The reproducibility of the assay was also proven by incurred sample reanalysis (ISR), which was conducted by selection of 125 subject samples in the vicinity of C_{max} and in the elimination phase of the pharmacokinetic profile of CIL and DCIL. The ISR results are represented in Supplementary Fig. 2. The % change for assay reproducibility in 125 incurred samples was within $\pm$13% for both the analytes, which was within the acceptance criterion of $\pm$20% [23].

3.6. Comparison with reported methods

The proposed UPLC−MS/MS method for CIL and DCIL is the most sensitive and rapid (analysis time for extraction and chromatography) compared to all other procedures for the determination of either CIL alone or with DCIL in human plasma. Compared with the two reported studies [8,10], the sensitivity obtained is 10 folds higher for CIL and DCIL. The present method employs small plasma volume (100 μL) for processing, which is at least five times lower than that of the existing methods. The on-column loading of CIL/DCIL at ULOQ was only 10/5 ng per sample injection, which was also much less than that of other procedures. Further, ISR study has been conducted for assay reproducibility which is not presented in other methods. A detailed comparison of the present method with reported procedures for CIL and DCIL in human plasma is given in Table 4.

4. Conclusion

The UPLC−MS/MS method for the simultaneous determination of CIL and DCIL in human plasma was developed and fully validated as per US FDA guidelines. The method offers several advantages over reported procedures, in terms of high sensitivity, low sample requirements, relatively simple extraction procedure and short overall analysis time. The efficiency of solid-phase extraction and a short chromatographic run time are highly favorable for high-throughput bioanalysis. Absence of matrix interference is effectively shown by post-column infusion and by the precision values for the slopes of calibration curves. The method is highly reproducible and is successfully used in the analysis of about 1500 samples in a clinical setting.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2014.08.001.

References

[1] US Department of Health and Human Services, Food and Drug Administration. NME Drug and New Biological Approval in 1999. Available from: ⟨http://www.fda.gov/…/howdrugsaredevelopedandapproved/drugandbiologicapprovalreports/ucm081686.htm⟩ (Last accessed April, 2014).

[2] Z.A. Al-Qudah, A.E. Hassan, A.L. Qureshi, Cilostazol in patients with ischemic stroke, Expert. Opin. Pharmacother. 12 (2011) 1305–1315.

[3] Y. Liu, Y. Shaku, J. Kambayashi, Phosphodi Esterases as Drug Targets, in: S.H. Francis, M. Conti, M.D. Houslay (Eds.), Hand-book of Experimental Pharmacology, Springer-Verlag, Berlin, Germany, 2011, p. 211.

[4] Y. Liu, Y. Shaku, M. Yoshitak, et al., Cilostazol (Pletal®): a dual inhibitor of cyclic nucleotide phosphodiesterase type 3 and adenosine uptake, Cardiovasc. Drug Rev. 19 (2001) 369–386.

[5] M. Hiratsuka, Y. Hinai, T. Sasaki, et al., Characterization of human cytochrome P450 enzymes involved in the metabolism of cilostazol, Drug Metab. Dispos. 35 (2007) 1730–1732.

[6] H. Akiyama, S. Kudo, T. Shimizu, The metabolism of a new antithrombotic and vasodilating agent, cilostazol, in rat, dog and man, Arzneimittelforschung 35 (1985) 1133–1140.

[7] H. Akiyama, S. Kudo, M. Odomi, et al., High-performance liquid chromatographic procedure for the determination of a new antithrombotic and vasodilating agent, cilostazol, in human plasma, J. Chromatogr. 338 (1985) 456–459.

[8] S.L. Bramer, P.N.V. Tata, S.S. Vengurlekar, et al., Method for the quantitative analysis of cilostazol and its metabolites in human plasma using LC/MS/MS, J. Pharm. Biomed. Anal. 26 (2001) 637–650.

[9] C.J. Fu, P.N.V. Tata, K. Okada, et al., Simultaneous quantitative determination of cilostazol and its metabolites in human plasma by high-performance liquid chromatography, J. Chromatogr. B 728 (1999) 251–262.

[10] R.V.S. Nirogi, V.N. Kandikere, M. Shukla, et al., Simultaneous quantification of cilostazol and its primary metabolite 3,4-dehydrocilostazol in human plasma by rapid liquid chromatography/tandem mass spectrometry, Anal. Bioanal. Chem. 384 (2006) 780–790.

[11] P.N.V. Tata, C.H.J. Fu, S.L. Bramer, Determination of cilostazol and its metabolites in human urine by high performance liquid chromatography, J. Pharm. Biomed. Anal. 24 (2001) 381–389.

[12] P.N.V. Tata, C.H.J. Fu, N.J. Browder, et al., The quantitative determination of cilostazol and its four metabolites in human liver microsomal incubation mixtures by high-performance liquid chromatography, J. Pharm. Biomed. Anal. 18 (1998) 441–451.

[13] K.J. Yeon, Y.J. Park, K.M. Park, et al., High performance liquid chromatographic analysis of cilostazol, in human plasma with on-line column switching, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 109–120.

[14] K.V.S. Varanasi, V. Sridhar, S. Potharaju, et al., Development and validation of a liquid chromatography/tandem mass spectrometry

assay for the simultaneous determination of nateglinide, cilostazol and its active metabolite 3,4-dehydro-cilostazol in Wistar rat plasma and its application to pharmacokinetic study, J. Chromatogr. B 865 (2008) 91–98.

[15] M.S. Attia, W.H. Mahmoud, A.O. Youssef, et al., Cilostazol determination by the enhancement of the green emission of Tb^{+3} optical sensor, J. Flouresc. 21 (2011) 2229–2235.

[16] FDA, Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), and Centre for Veterinary Medicine (CVM), 2001.

[17] D.P. Patel, P Sharma, M. Sanyal, et al., Challenges in the simultaneous quantitation of sumatriptan and naproxen in human plasma: application to a bioequivalence study, J. Chromatogr. B 902 (2012) 122–131.

[18] B.K. Matuszewski, Standard line slopes as a measure of relative matrix effect in quantitative HPLC-MS bioanalysis, J. Chromatogr. B 830 (2006) 293–300.

[19] FDA, Guidance for Industry: ICH E6 Good Clinical Practice. US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), 1996.

[20] S.M. Chesnut, J.J. Salisbury, The role of UHPLC in pharmaceutical development, J. Sep. Sci. 30 (2007) 1183–1190.

[21] S. Bansal, A. DeStefano, Key elements of bioanalytical method validation for small molecules, AAPS J. 9 (2007) E109–E114.

[22] S.K. Woo, W.K. Kang, K.I. Kwon, Pharmacokinetic and pharmacodynamic modeling of the antiplatelet and cardiovascular effects of cilostazol in healthy humans, Clin. Pharmacol. Ther. 71 (2002) 246–252.

[23] M. Yadav, P.S. Shrivastav, Incurred sample reanalysis (ISR): a decisive tool in bioanalytical research, Bioanalysis 3 (2011) 1007–1024.