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Method for the quantitative analysis of cilostazol and its metabolites in human plasma using LC/MS/MS

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Abstract

An LC/MS/MS method for the simultaneous determination of cilostazol, a quinolinone derivative, and three active metabolites, OPC-13015, OPC-13213, and OPC-13217, in human plasma was developed and validated. Cilostazol, its metabolites, and the internal standard, OPC-3930 were extracted from human plasma by liquid–liquid partitioning followed by solid-phase extraction (SPE) on a Sep-Pak™ silica column. The eluent from the SPE column was then evaporated and the residue reconstituted in a mixture of methanol/ammonium acetate buffer (pH 6.5) (2:8 v/v). The analytes in the reconstituted solution were resolved using reversed-phase chromatography on a Supelcosil LC-18-DB HPLC column by an 17.5-min gradient elution. Cilostazol, its metabolites, and the internal standard were detected by tandem mass spectrometry with a Turbo Ionspray™ interface in the positive ion mode. The method was validated over a linear range of 5.0–1200.0 ng/ml for all the analytes. This method was demonstrated to be specific for the analytes of interest with no interference from endogenous substances in human plasma or from several potential concomitant medications. For cilostazol and its metabolites, the accuracy (relative recovery) of this method was between 92.1 and 106.4%, and the precision (%CV) was between 4.6 and 6.5%. During the validation, standard curve correlation coefficients equalled or exceeded 0.999 for cilostazol and its metabolites. These data demonstrate the reliability and precision of the method. The method was successfully cross-validated with an established HPLC method.

Keywords: Cilostazol; LC/MS/MS; Human plasma

1. Introduction

Cilostazol [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone], also known as OPC-13013 and Pletal®, is a novel quinolinone derivative which is indicated for the

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treatment of intermittent claudication [1,2]. Cilostazol is extensively metabolized [3]. The available analytical methods either quantitated cilostazol only [4], lacked adequate specificity from endogenous or xenobiotic interferences [4–6], or required long chromatographic run times [6]. Therefore, an LC/MS/MS method was developed and validated for the simultaneous quantitation of cilostazol and three metabolites: OPC-13015, OPC-13213, and OPC-13217 in human plasma. This method offers the advantages of a relatively short run time with excellent specificity and improved sensitivity. This method has been used to support the clinical development of cilostazol. The LC/MS/MS method was cross-validated to an existing HPLC method [6] and found to give comparable results.

2. Experimental

2.1. Reagents

HPLC-grade methanol, chloroform, methyl-*t*-butyl ether, acetonitrile, and analytical grade glacial acetic acid and ammonium acetate were purchased from Fisher Scientific, Inc. (Pittsburgh, PA, USA). High purity ($>99.0\%$) cilostazol, OPC-13015, OPC-13213, OPC-13217, and OPC-3930 (internal standard), were obtained from Otsuka Pharmaceutical Co. (Japan). The Chem-Elute CE #1003™ (12 cc) SPE columns were obtained from Varian Sample Preparation Products (Harbor City, CA, USA), and the Sep-Pak™ Silica 200 mg SPE columns were obtained from Waters Associates (Milford, MA, USA). Blank human plasma (sodium heparin anticoagulant) was obtained from commercial vendors.

2.2. Instrumentation

For the LC/MS/MS analysis, the chromatographic system consisted of two Shimadzu Model LC-10AT pumps, a Shimadzu SCL-10A system controller, a Shimadzu SIL-10AXL autosampler, and a Supelcosil LC-18-DB column (250×2.1 mm i.d., 5 μm, Supelco) preceded by a C-18 (Waters Associates) guard column. The column effluent was analyzed on a PE-Sciex API III Plus™ Mass Spectrometer with a Turbo Ionspray (electrospray) interface. The specific precursor and product ions for each analyte were monitored. For the comparitor HPLC analysis, the same HPLC system was used, except for addition of a Shimadzu SPD-10A UV detector (254 mm). The analytical column was a TosoHaas TSK-GEL ODS-80TM (150×4.6 mm, 5 μm).

2.3. Analytical standards

Primary stock solutions of each compound were prepared in methanol. Working solutions containing cilostazol, OPC-13015, OPC-13213, and OPC-13217 were prepared by combining aliquots of each primary solution and diluting with blank human plasma. The working solution for the internal standard was prepared by diluting an aliquot of the primary solution with HPLC-grade water.

2.4. Preparation of calibration standards and quality control samples

Human plasma calibration standards of cilostazol and metabolites (5.0, 10.0, 20.0, 60.0, 120.0, 200.0, 400.0, 800.0, and 1200.0 ng/ml) were prepared by spiking the working standard solutions into drug-free human plasma containing sodium heparin as the anticoagulant. Quality Control (QC) samples (at 15.0, 50.0, 500.0, and 1000.0 ng/ml) were prepared in a similar manner from separate primary and working stock solutions.

2.5. Sample extraction

The sample preparation involved minor changes to the published method [6]. Calibration standards and QC samples containing 0.5 ml human plasma spiked with analytes of interest, 50 μl of the working internal standard, and 250 μl of 100 mM ammonium sulfate solution (pH 7.0) were added to 15-ml glass screw top tubes and mixed with 5 ml of *n*-butanol/chloroform/methyl *t*-butyl ether (3:20:77 v/v/v). The samples were vortex mixed on a Multi-Tube Vortexer (VWR) for 10 s; centrifuged at 2500 rev./min for 5 min in

a bench top centrifuge (Jouan), and the organic layers were removed by freezing the aqueous layers in a dry ice/acetone bath and decanting the organic layers to clean glass tubes. The organic portions were mixed with 0.5 ml of 0.1 M ammonium acetate (pH 6.5) and evaporated under nitrogen at 35°C (Turbo Vap™, Zymark) to about 1.0 ml. The aqueous layers were placed on preconditioned Chem Elute CE # 1003 SPE columns connected on top of Sep-Pak silica columns and washed first with 6.0 ml of chloroform followed by a 3.0 ml chloroform wash using gravity elution. The Chem Elute columns were removed and the analytes were then eluted from the Sep-Pak columns with 2.5 ml of a chloroform/methanol mixture (50/50 v/v) by gravity elution. The combined extracts were evaporated under nitrogen at 40°C. The residues were dissolved in 200 μl of methanol and 800 μl of 0.1 M ammonium acetate (pH 6.5) was added. Twenty microliters of these reconstituted solutions were injected onto the LC/MS/MS system for analysis.

Table 1
HPLC gradient program for the determination of cilostazol and its metabolites

Time (min)	% Pump B
0.01	
1.00	20
2.00	35
3.50	65
7.50	65
7.50	20
17.50	STOP

Table 2
Specific precursor and product ions plus retention times for cilostazol, OPC-13015, OPC-13213, OPC-13217, and OPC-3930

Compound	Precursor → product ion transition (m/z)	Approximate retention time (min)
Cilostazol	370.2 → 288.2	11.8
OPC-13015	368.2 → 286.2	11.4
OPC-13213	386.2 → 288.2	5.9
OPC-13217	386.2 → 288.2	7.0
OPC-3930	354.3 → 272.0	10.6

2.6. Chromatographic conditions

Analysis was performed by reverse-phase HPLC. Mobile phase A consisted of acetonitrile/2 mM ammonium acetate–0.1% acetic acid buffer (10/90 v/v) and mobile phase B consisted of acetonitrile/2 mM ammonium acetate–0.1% acetic acid buffer (90:10 v/v) at a flow rate of 1.0 ml/min. For the LC/MS/MS method, the gradient program used to separate cilostazol and its three metabolites is presented in Table 1. The percent of the mobile phase provided by pump B was increased step-wise from 20 to 65% over 7.5 min. The mobile phase returned to the original conditions at 7.5 min and was allowed to remain at that ratio for an additional 10 min for elution of OPC-13013, OPC-13015, and the interval standard, plus re-equilibration. The MS scan parameters included an interface temperature of 70°C, auxiliary gas flow (N_2) of 7 l/min, nebulizer gas pressure (N_2) of 40 psi, curtain gas (N_2) of 1.2 l/min, nebulizer temperature of 510°C, dwell time of 500 ms, and pause time of 1 ms. The components of the eluent were identified and quantified by retention time and specific precursor/product ion transitions in the mass spectrometer (Table 2).

For the HPLC/UV method, the solvent gradient increased from 27% pump B to 100% pump B over 40 min and returned to 27% for 5 min to re-equilibrate.

2.7. Calibration curve and calculations

After a batch of calibration standards and QC samples were chromatographed and peak responses for analytes and the internal standard were measured, a calibration curve was generated for each analyte. The regression parameters of slope, intercept, and correlation coefficient were calculated using weighted ($1/x$) linear regression. The concentrations of calibration standards were back-calculated using the following equation to measure residuals and standard errors to evaluate the validity of the method:

$$\text{Analyte concentration} = \frac{(\text{Analyte peak height/IS peak height}) - \text{Intercept}}{\text{Slope}}$$

QC samples were analyzed in each analytical run along with other samples. The calculated concentration for these QC samples was also computed using the above equation. The LC/MS/MS data were acquired using the MacQuan data acquisition software. The data were subsequently imported into Microsoft Excel (version 5.0) for routine computations.

2.8. Assessment of validation

To evaluate the precision and accuracy of the assay for cilostazol and its metabolites in human plasma, calibration standards were prepared by spiking cilostazol and metabolites into drug-free blank human plasma at concentrations between 5.0 and 1200.0 ng/ml. QC samples were prepared by spiking cilostazol and metabolites into drug-free blank human plasma at 15.0, 50.0, 500.0 and 1000.0 ng/ml. Batches, consisting of three calibration standards at each concentration were analyzed on three different days to complete the method validation. In each batch, QC samples at each concentration were assayed in sets of five replicates to evaluate the intra- and inter-day accuracy and precision. In addition, QC samples at 50.0, 500.0, and 1000.0 ng/ml were subjected to three freeze/thaw cycles to study the stability of all the analytes. Also, QC samples at the highest concentration were diluted with blank human plasma prior to extraction to evaluate the effect of sample dilution on accuracy and precision. To evaluate the long-term storage stability of analytes in human plasma, samples from the QC sample pools were analyzed against freshly prepared calibration standards, first on the day of preparation and then after storing at -20°C for at least 2 months.

3. Results

3.1. Specificity

Liquid chromatography conditions were optimized so that cilostazol, its metabolites, and the internal standard were satisfactorily separated. The precursor/product ion transitions and retention times for cilostazol, its metabolites, and internal standard are in Table 2. The tandem MS detection mode uses specific precursor/product ion transitions, except for the isomers OPC-13213 and OPC-13217. These metabolites were physically separated by the chromatography. The product ion scan for cilostazol is shown in Fig. 1. The structure depicting the loss of the cyclohexane ring in obtaining the 288.2 m/z product ion is included. The comparable product ions were also chosen for the other analytes. These steps, combined with the extensive sample extraction process improved specificity. Blank human plasma, not spiked with any analytes, showed no peaks that significantly interfered with either cilostazol, OPC-13015, OPC-13213, OPC-13217, or the internal standard. Fig. 2 shows a typical LC/MS/MS scan for a blank human plasma sample with no analytes. Fig. 3 shows a typical LC/MS/MS scan for plasma containing cilostazol, its metabolites and the internal standard (Calibration Standard). Several potential concomitant medications were screened to assure method specificity. No significant influence on analytes' extraction efficiency or analytical performance was observed when erythromycin (4 μg/ml), lovastatin and metabolite (100 ng/ml), clopidogrel (100 ng/ml), or diltiazem and metabolites (200 ng/ml) were present in human plasma. Sample carryover effect was not observed as evidenced by the absence of MS/MS response in the regions of the peaks of interest for blank human plasma extracts randomly placed throughout the analytical run.

3.2. Linearity

Linearity was observed over the range between 5.0 and 1200.0 ng/ml for cilostazol and its three metabolites as shown in Table 3. The mean correlation coefficients were 0.999 for cilostazol and its metabolites. In addition, acceptable accuracy and precision were obtained over the entire calibration curve range for each compound (Table 4). These data clearly demonstrated the linearity of the method.

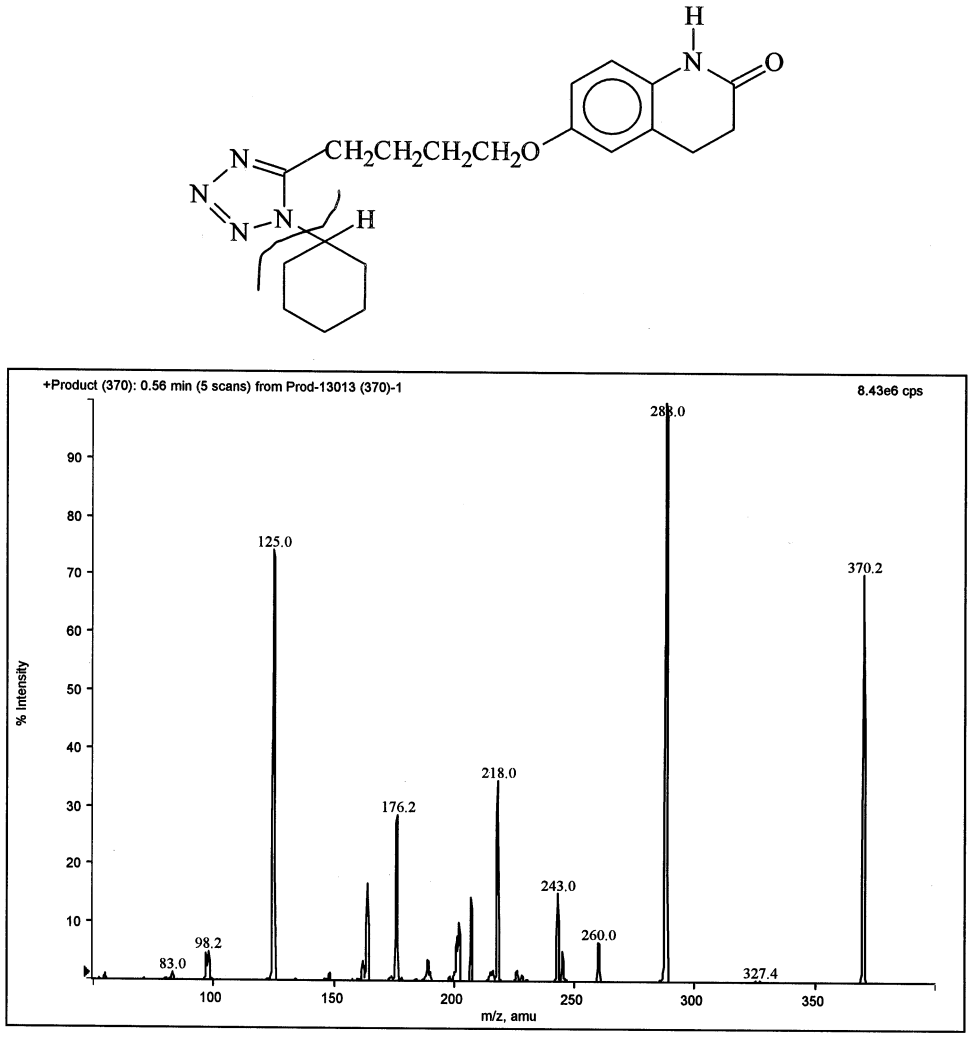


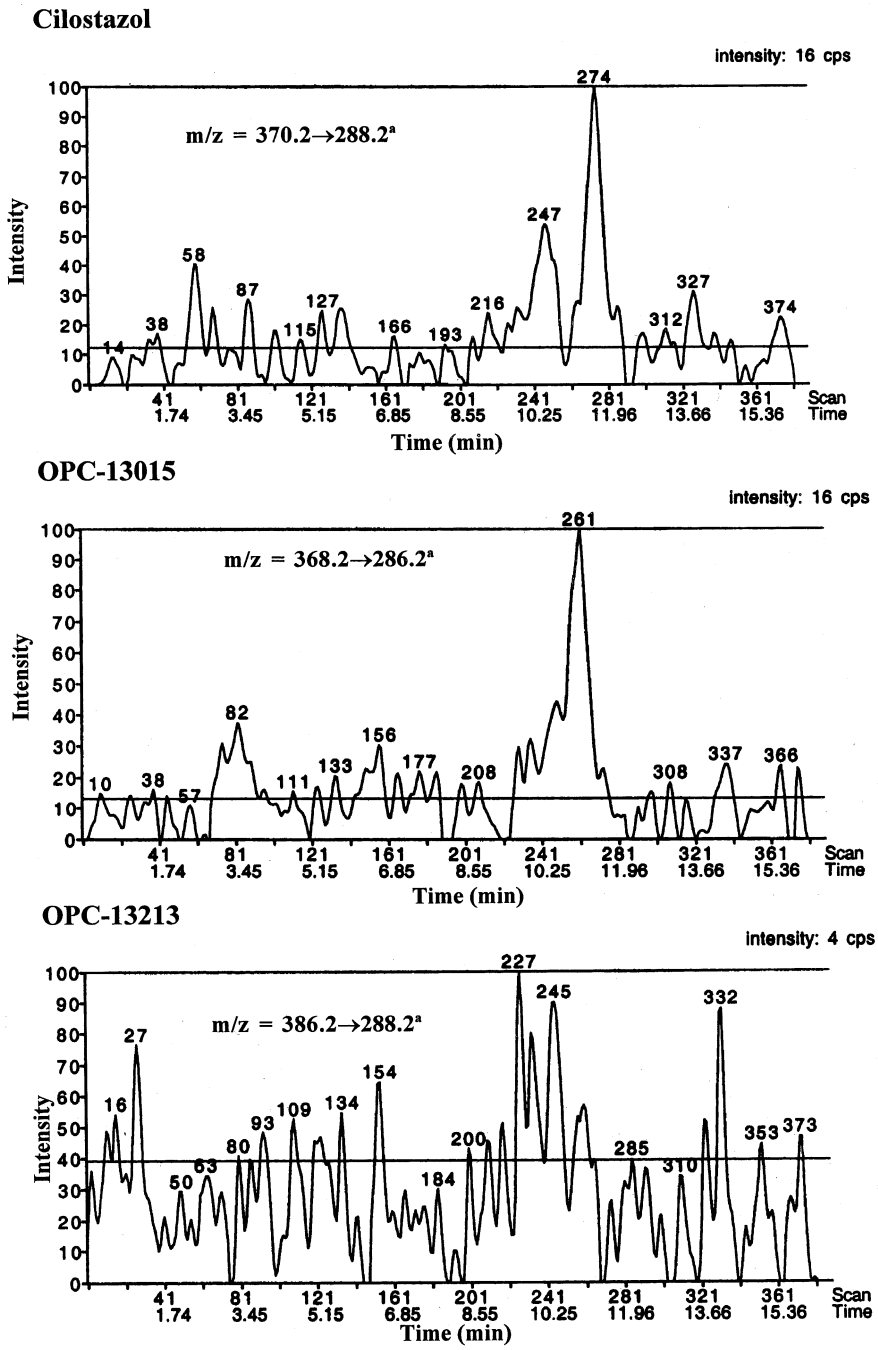
Fig. 1. Structure of cilostazol depicting the loss of the cyclohexane ring during collision-induced dissociation.

3.3. Sensitivity

The lower limit of quantitation (LLOQ) was set at 5.0 ng/ml for cilostazol and its metabolites using 0.5 ml of human plasma. Fig. 4 presents a typical chromatogram of spiked human plasma sample containing 5.0 ng/ml of cilostazol and metabolites. The peak heights for all analytes are at least three times the background noise. Table 4 presents the performance of calibration standards at this concentration. The mean percent accuracy values were 89.6% for cilostazol, 90.0% for OPC-13015, 94.0% for OPC-13213, and 97.4% for OPC-13217 with coefficients of variation (%CV) of 7.4, 7.1, 11.5, and 8.0%, respectively.

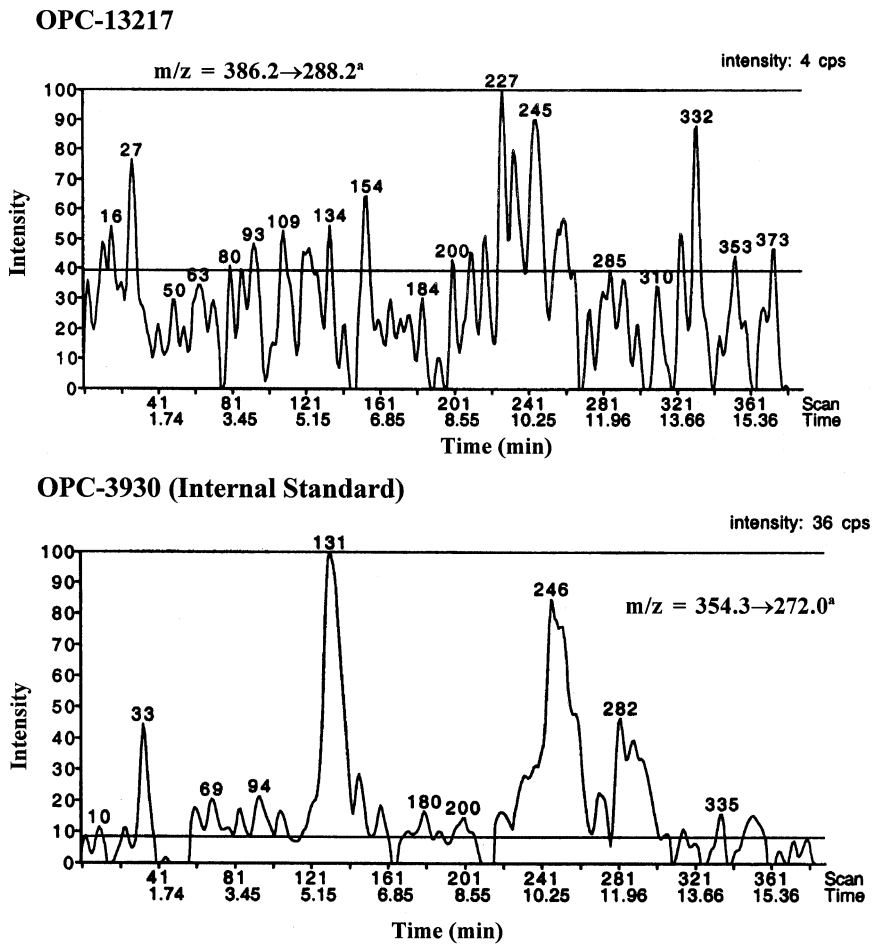
3.4. Extraction recovery

The mean extraction recoveries were 101.0, 106.6, 88.2, 94.6, and 102.3% for cilostazol, OPC-13015, OPC-13213, OPC-13217, and OPC-3930, respectively.



[a] m/z = precursor ion → product ion

Fig. 2. Representative LC-MS/MS ion chromatograms of a blank human plasma sample.



[a] m/z = precursor ion → product ion

Fig. 2. (*Continued*)

3.5. Inter-batch and intra-batch accuracy and precision

QC samples containing cilostazol and metabolites were prepared and analyzed along with other samples during the validation. Table 5 presents a summary of intra- and inter-batch precision and accuracy. The intra-batch accuracy for cilostazol and its metabolites ranged between 86.3 and 114.3% at four different concentration with the precision (%CV) between 1.4 and 8.2%. The inter-batch accuracy for cilostazol and its metabolites ranged between 89.3 and 111.2% at four different concentrations with %CV between 3.0 and 8.3%.

3.6. Stability

Solution stability was assessed by comparing stored solutions to freshly prepared comparable solutions. Prepared solutions, such as system validation solutions (in ammonium acetate/methanol) and working solutions (8% methanol in plasma) showed no evidence of degradation for cilostazol, its three metabolites, or the internal standard OPC-3930 over the period of the validation. Sample processing was found to require approximately 7 h for a typical batch of 48–72 samples. No significant degradation was observed for any analytes of interest during the sample processing.

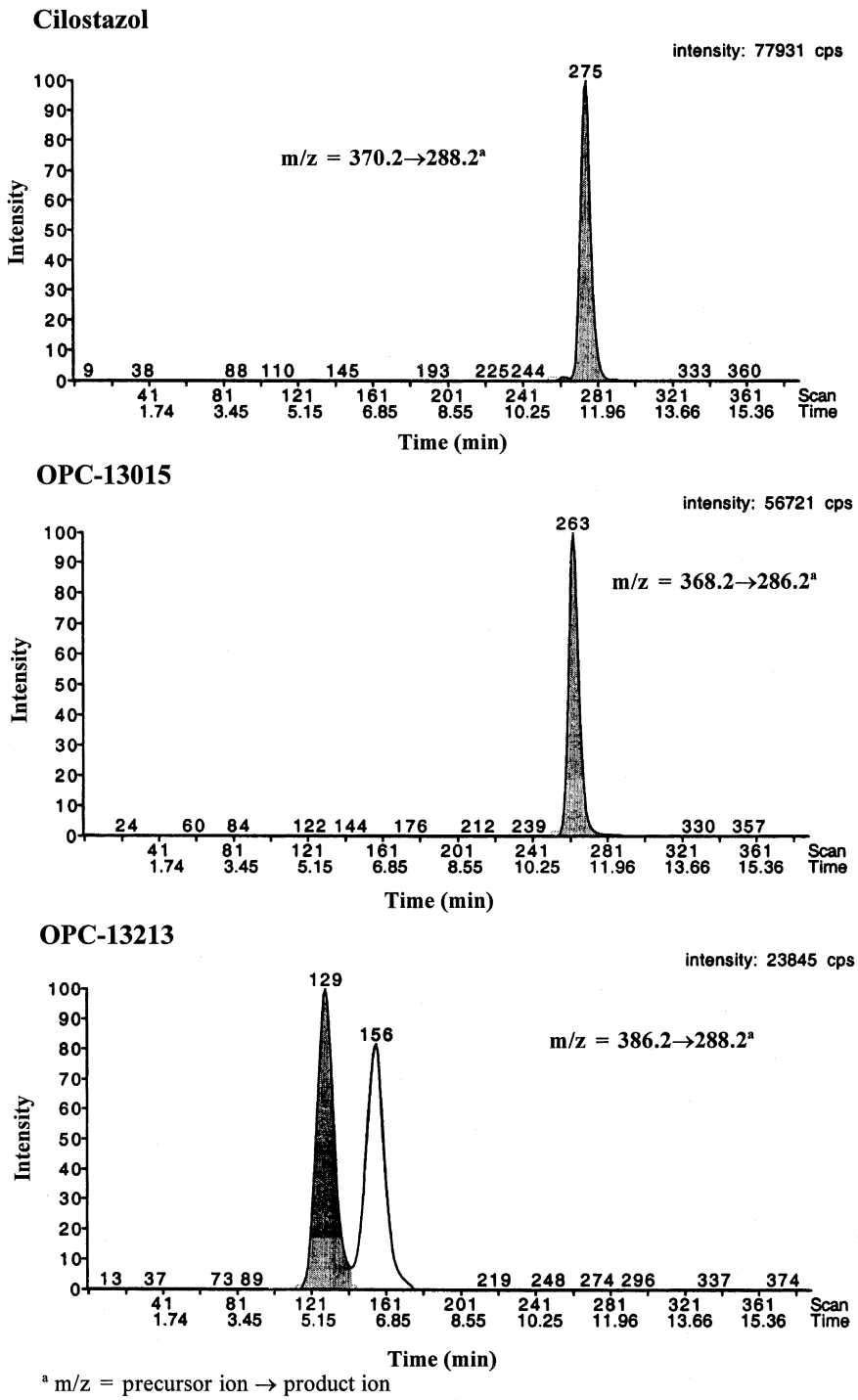


Fig. 3. Representative LC-MS/MS ion chromatograms for the ≈1200 ng/ml standard in human plasma (highest calibration curve point).

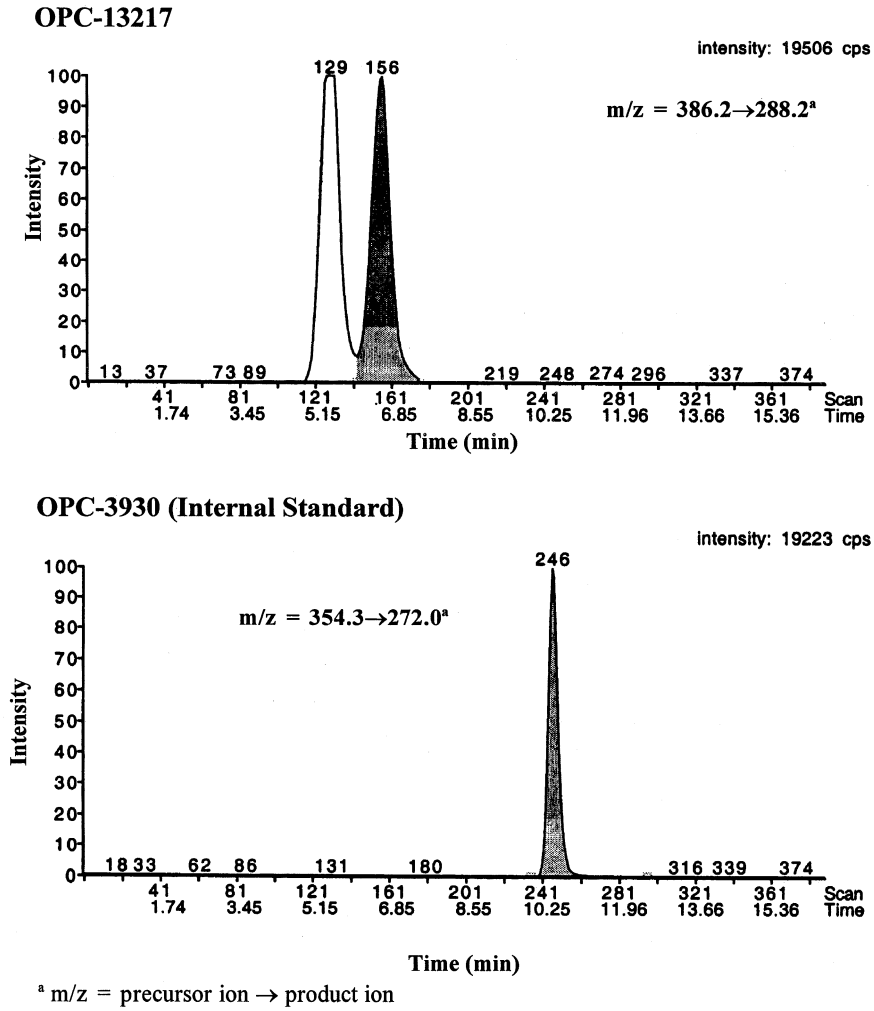


Fig. 3. (*Continued*)

Table 3
Regression parameters for cilostazol and metabolite calibration curves in human plasma (5.0–1200.0 ng/ml)

Analyte	Slope ($\times 10^{-5}$)[a]	Intercept ($\times 10^{-5}$)[a]	Correlation coefficient[a]
Cilostazol	29.66 ± 5.30	50.47 ± 38.8	0.999
OPC-13015	22.00 ± 2.84	39.60 ± 24.1	0.999
OPC-13213	16.07 ± 1.80	13.31 ± 11.2	0.999
OPC-13217	15.63 ± 0.90	6.61 ± 14.43	0.999

[a] Mean ± S.D., $n = 9$.

Cilostazol and its metabolites were stable (less than 15% degradation) in human plasma at room temperature for at least 24 h. The autosampler stability for the reconstituted extract was at least 54 h either at room temperature or at 4°C. No significant degradation was observed for cilostazol

and its metabolites in human plasma samples when subjected to three freeze–thaw cycles. During the validation, cilostazol, OPC-13015, OPC-13213, and OPC-13217 were stable for at least 2 months when stored at −20°C. Subsequently, plasma samples collected from clinical studies of cilostazol were analyzed and the QC samples performance demonstrated that cilostazol and its metabolites were stable in human plasma samples when stored at −20°C for at least 12.5 months. The process stability and storage stability were assessed by measurement of calculated concentration versus nominal concentration against freshly prepared calibration standard curves.

3.7. Dilution

To evaluate the sample dilution procedures, 0.25- or 0.05-ml aliquots of the QC samples were diluted two- or 10-fold with drug-free blank plasma prior to extraction. The data indicated that the relative recoveries of cilostazol and three metabolites ranged from 96.4 to 109.5% with precision of 1.2–7.9%. The validation of the 10-fold dilution procedure extended the upper limit of quantitation for this analytical method to 12 000 ng/ml.

3.8. Comparison of LC/MS/MS method with the HPLC–UV method

For the HPLC method, a 45-min run time was required to separate cilostazol, its metabolites, and the internal standard from each other [6]. The LC/MS/MS method reduced the run time to 17.5 min with improved specificity and sensitivity. To compare clinical results obtained by the two methods, 60 plasma samples collected from subjects administered cilostazol orally were analyzed using both the HPLC method and the LC/MS/MS method. Fig. 5 demonstrates adequate agreement between the methods for cilostazol (slope = 1.042, $r^2 = 0.988$). Comparable results were obtained for OPC-13015 and OPC-13213. Too few samples had measurable plasma OPC-13217 concentrations to do the method comparison.

4. Discussion

The results from non-clinical studies and clinical studies indicated that cilostazol is extensively metabolized [3,6]. However, only a few metabolites are detectable in human plasma and are pharmacologically active (OPC-13015, OPC-13213, and OPC-13217). The previous HLPC methods [4–6] were adequate for studies in normal volunteers but had limitations in specificity when used to analyze samples from typical patients taking various concomitant medications. The elaborate extraction procedure, gradient elution chromatography, and tandem mass spectral analysis provided a high degree of specificity. In addition, the new method increased sensitivity from 20.0 down to 5.0 ng/ml. The goal was to develop and validate an LC/MS/MS method to quantitate cilostazol and the three known major circulating metabolites in human plasma. OPC-3930 was chosen to be the internal standard based on earlier experience [5,6].

Various extraction procedures were evaluated, for sample preparation prior to chromatography.

Table 4
Calculated concentrations of cilostazol and metabolites in calibration standards prepared in human plasma (5.0–1200.0 ng/ml)[a]

Analyte	5.0 ng/ml	10.0 ng/ml	20.0 ng/ml	120.0 ng/ml	400.0 ng/ml	800.0 ng/ml	1200.0 ng/ml
Cilostazol	4.48 ± 0.33	9.86 ± 0.66	20.9 ± 0.6	126 ± 9.8	420 ± 22.0	794 ± 33.1	1180 ± 43.3
OPC-13015	4.50 ± 0.32	9.94 ± 0.62	20.6 ± 0.8	126 ± 7.8	416 ± 17.5	795 ± 33.6	1184 ± 47.1
OPC-13213	4.70 ± 0.54	9.93 ± 0.90	20.5 ± 1.1	124 ± 8.3	404 ± 32.2	805 ± 51.8	1187 ± 81.1
OPC-13217	4.87 ± 0.39	10.1 ± 0.69	19.9 ± 1.3	122 ± 7.6	403 ± 28.5	810 ± 48.7	1186 ± 73.6

[a] Values are mean ± S.D.; $n = 9$.

Table 5
Precision, accuracy and sensitivity of cilostazol and its metabolites in human plasma quality control samples

Analyte	Statistical variable	Intra-batch ($n = 5$)				Inter-batch ($n = 15$)			
		15.0 (ng/ml)	50.0 (ng/ml)	500.0 (ng/ml)	1000.0 (ng/ml)	15.0 (ng/ml)	50.0 (ng/ml)	500.0 (ng/ml)	1000.0 (ng/ml)
Cilostazol	Mean	13.7–15.6	52.7–56.4	506–533	977–1078	14.5	53.9	519	1022
	%CV	3.9–8.2	1.9–2.9	2.8–3.9	2.3–6.3	8.3	3.8	3.9	5.8
	% Accuracy	91.6–103.8	105.5–112.8	101.3–106.6	97.7–107.8	96.9	107.9	103.9	102.2
OPC-13015	Mean	14.6–16.1	54.14–57.16	529–543	1027–1083	15.3	55.6	535	1056
	%CV	5.5–8.2	1.7–3.9	1.4–4.5	1.8–5.6	7.5	3.7	3.0	4.1
	% Accuracy	97.6–107.1	108.3–114.3	105.8–108.5	102.7–108.3	101.9	111.2	106.9	105.6
OPC-13213	Mean	12.9–13.8	44.1–52.2	451–499	883–926	13.5	47.7	468	893
	%CV	1.4–6.9	2.8–4.6	2.3–5.5	2.2–7.6	5.3	8.1	6.4	5.5
	% Accuracy	86.3–91.8	88.1–104.4	90.1–99.8	88.3–92.6	89.9	95.5	93.6	89.3
OPC-13217	Mean	15.6–16.7	46.3–54.7	488–543	950–1016	16.3	50.1	507	978
	%CV	3.4–6.5	1.7–3.3	1.9–6.0	3.1–7.2	6.2	7.5	6.7	5.6
	% Accuracy	104.2–111.4	92.6–109.4	97.5–108.5	95.0–101.6	108.9	100.2	101.3	97.8

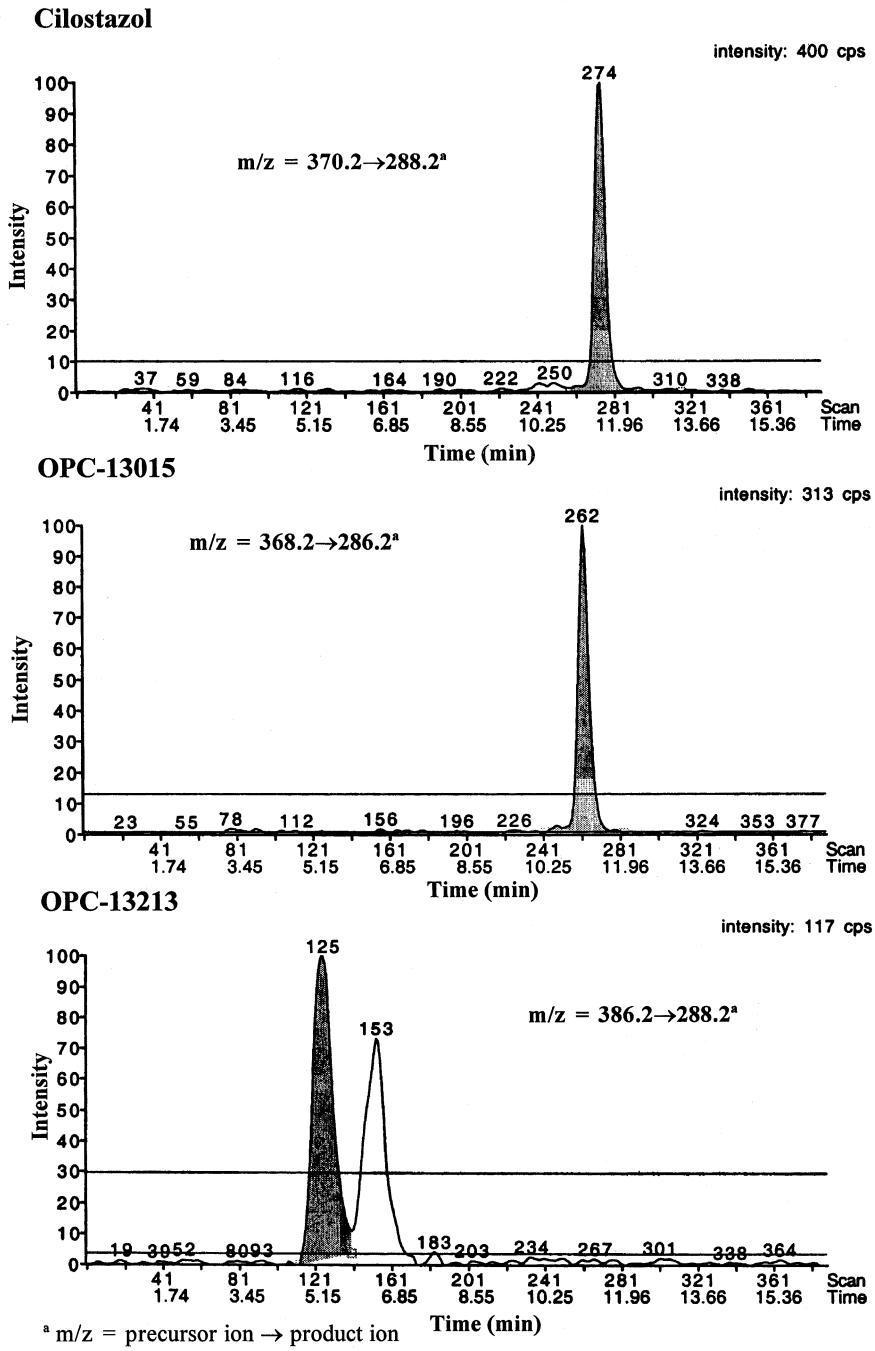
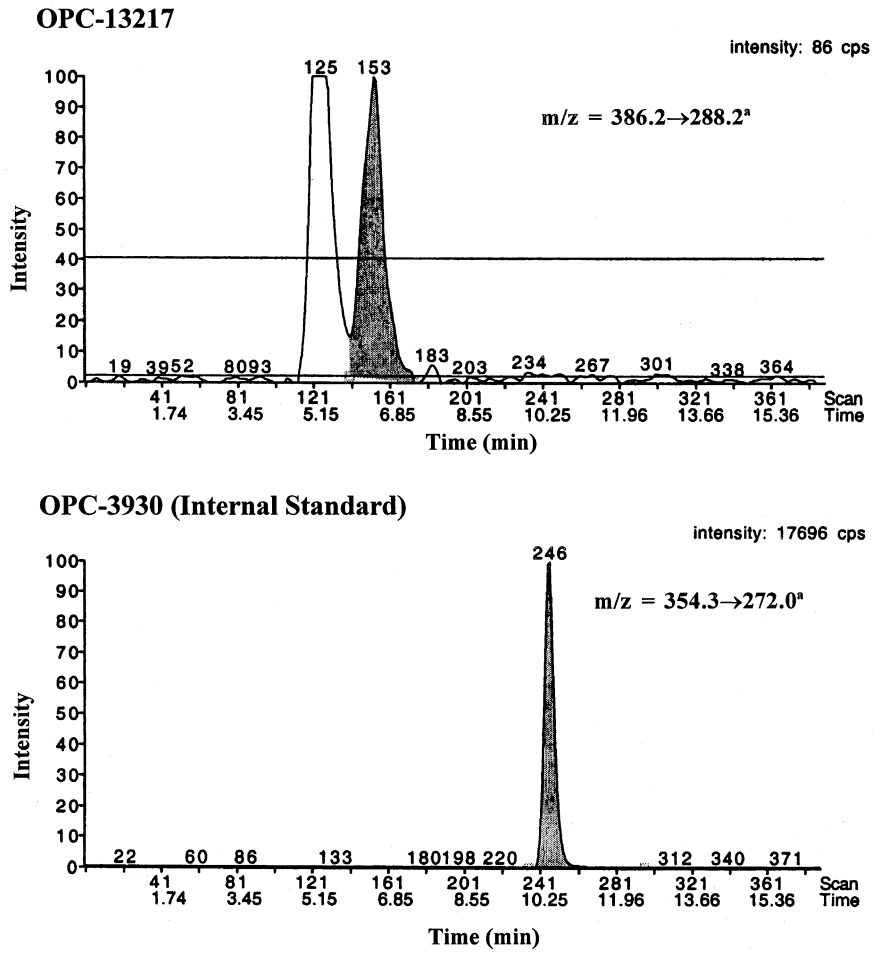


Fig. 4. Representative LC-MS/MS ion chromatograms for the ≈5 ng/ml standard in human plasma (lowest calibration curve point).



[a] m/z = precursor ion → product ion

Fig. 4. (*Continued*)

The previously developed extraction procedure [6] was modified slightly to optimize recovery and improve sample reconstitution following evaporation of solvent.

Several HPLC columns with different packing materials and different lengths were evaluated. The Supelcosil LC-18-DB column was found to achieve the best resolution among cilostazol, its metabolites, the internal standard and endogenous background from human plasma. The chromatographic run time of 17.5 min per sample was a substantial improvement over the HPLC run time, but was still relatively time consuming. However, it was necessary to ensure the best specificity, resolution and selectivity for the analytes of interest.

The overall accuracy and precision for QC sample pools were within the $\pm 15\%$ range generally accepted for bioanalytical methods. Therefore, this method demonstrated adequate sensitivity, accuracy, precision, and linearity.

5. Conclusion

A reverse-phase HPLC method with positive ion MS/MS detection was developed and validated for

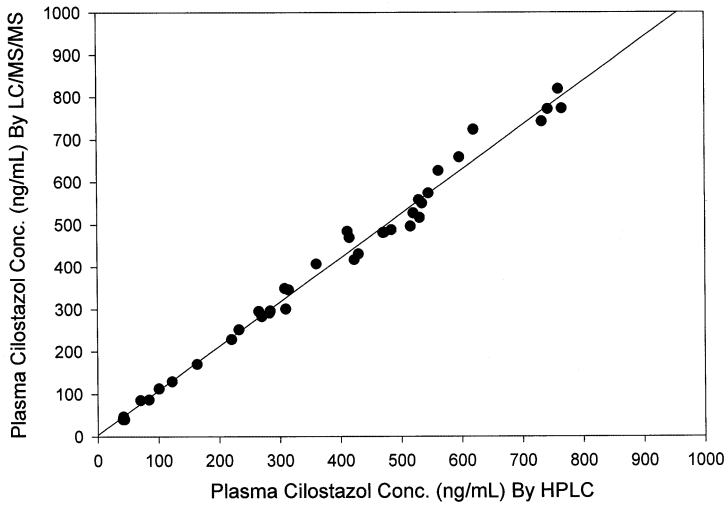


Fig. 5. Comparison of plasma cilostazol concentrations obtained using the LC/MS/MS and HPLC-UV methods of analysis.

the quantitation of cilostazol and three metabolites (OPC-13015, OPC-13213, and OPC-13217) in human plasma. The results of the method validation demonstrate acceptable accuracy and precision with adequate specificity and stability. The method has a lower limit of quantitation (LLOQ) of 5.0 ng/ml in human plasma samples. This increased sensitivity for cilostazol and metabolites was required for adequate pharmacokinetic evaluation of clinical studies. The LC/MS/MS method was found to achieve comparable precision and accuracy to the HPLC method, while offering the advantage of greater sensitivity and more rapid analysis of plasma samples with a high degree of specificity. Economically, the higher sample costs of LC/MS/MS are offset by the greater speed in completing projects, resulting in shorter time to market.

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