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The quantitative determination of cilostazol and its four metabolites in human liver microsomal incubation mixtures by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatography-ultraviolet (HPLC-UV) method for the quantitation of cilostazol and four of its principal metabolites (i.e. OPC-13015, OPC-13213, OPC-13217 and OPC-13326) in human liver microsomal solutions was developed and validated. Cilostazol, its metabolites, and the internal standard (OPC-3930), were analyzed by protein precipitation followed by reverse-phase HPLC separation on a TSK-Gel ODS-80TM (150 × 4.6 mm, 5 μm) column and a Cosmil C-18 column (150 × 4.6 mm, 5 μm) in tandem and UV detection at 254 nm. An 80 min gradient elution of mobile phase acetonitrile in acetate buffer (pH = 6.50) was used to obtain quality chromatography and peak resolution. All the analytes were separated from each other, with the resolution being 2.43–17.59. The components of liver microsomal incubation mixture and five metabolic inhibitor probes (quinidine sulfate, diethyl dithiocarbamate (DEDTC), omeprazole, ketoconazole and furafylline) did not interfere with this analytical method. The LOQ was 1000 ng ml^{-1} for cilostazol and 100 ng ml^{-1} for each of the metabolites. This method has been validated for linear ranges of 100–4000 ng ml^{-1} for OPC-13213, OPC-13217 and OPC-13326; 100–2000 ng ml^{-1} for OPC-13015; and 1000–20000 ng ml^{-1} for cilostazol. The percent relative recovery of this method was established to be 81.2–101.0% for analytes, with the precision (% coefficient of variation (CV)) being 2.8–7.7%. The autosampler stability of the analytes was evaluated and it was found that all analytes were stable at room temperature for a period of at least 17 h. This assay has been shown to be precise, accurate and reproducible.

Keywords: Cilostazol; High-performance liquid chromatography; Gradient elution; Liver microsomes

1. Introduction

Cilostazol (OPC-13013, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H-quinolin-

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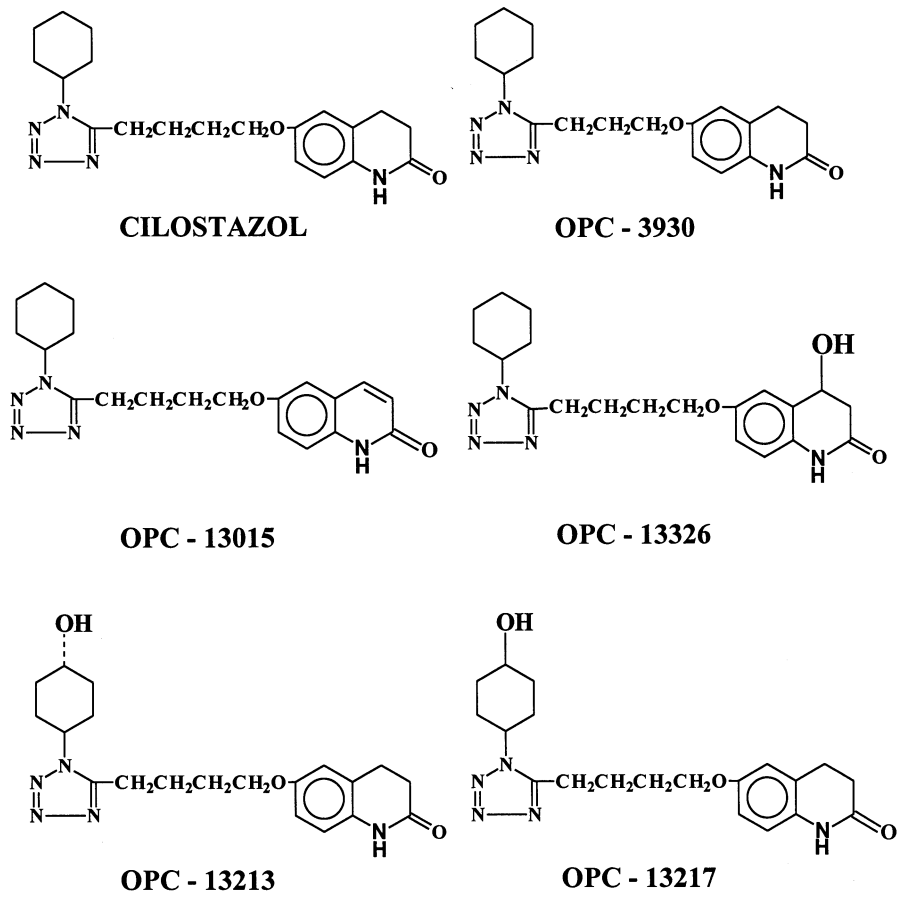

Fig. 1. Chemical structures of cilostazol and its major metabolites.

one)), is a new quinolinone derivative which is currently under investigation for use in the treatment of intermittent claudication [1,2]. Cilostazol has been reported to be extensively metabolized to at least 11 metabolites [3]. However, the primary metabolites of interest are OPC-13015 (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-2(1H)-quinolinone); OPC-13213 (3,4-dihydro-6-[4-[1-(*trans*-4-hydroxycyclohexyl)-1H-tetrazol-5-yl) butoxy-2(1H)-quinolinone); OPC-13217 (3,4-dihydro-6-[4-[1-(*cis*-4-hydroxycyclohexyl)-1H-tetrazol-5-yl)butoxy-2(1H)-quinolinone); and OPC-13326 (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-4-hydroxy-2(1H)-quinolinone) (Fig. 1). Currently published analytical methods used to assess plasma or urine [3,4] could not be applied to the investigation of cilostazol metabolism because these available analytical methods quantitate only cilostazol and require extensive sample pretreatment. Hence, a practical high-performance liquid chromatography (HPLC) method was developed for cilostazol and its metabolites (OPC-13015, OPC-13213, OPC-13217 and OPC-13326) in liver microsomal incubation mixtures to support the in vitro human liver metabolism studies of cilostazol. This HPLC method was validated following the guidelines suggested by the International Committee on Harmonization (ICH) and United State Pharmacopeia (USP) [6,7].

2. Experimental

2.1. Reagents

Cilostazol (OPC-13013), OPC-13015, OPC-13213, OPC-13217, OPC-13326 and the internal standard OPC-3930 (~ 98% pity) were obtained from the Tokushima Research Institute, Otsuka America Pharmaceutical Co. (Tokushima, Japan). HPLC-grade methanol and acetonitrile were purchased from Burdick and Jackson Laboratories (Muskegon, MI, USA) and glacial acetic acid and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was collected daily for analytical use and tested periodically for its compliance with compendial standards. All chemicals were of analytical reagent grade or of known analytical purity.

2.2. Instrumentation

The gradient HPLC system consisted of two Shimadzu LC-10AS pumps, a Shimadzu SCL-10A controller, a Shimadzu SIL-10A autosampler, a 5 μm TSK-GEL ODS-80TM column (150 × 4.6 mm i.d., 5 μm, Tosohaas Bioseparation Specialists) and a Cosmosil C18 column (150 × 4.6 mm i.d. 5 μm, Nacalai Tesque) in tandem, a Shimadzu SPD-6A ultraviolet absorbance detector and a Shimadzu Model CR-501 integrator. The wavelength of the detector was set at 254 nm.

2.3. Analytical standards

A stock standard solution consisting of cilostazol (2000 μg ml^{-1}) and its four metabolites (200 μg ml^{-1}) was prepared with methanol. The stock standard solution of the internal standard, OPC-3930 (100 μg ml^{-1}), was also prepared in methanol. The working solution of internal standard (10 μg ml^{-1}) was prepared by dilution with HPLC-grade water. All stock solutions were stored at 4°C.

2.4. Calibration standards

Calibration standards in the microsomal incubation mixture at concentrations of 1, 2, 6, 10, 15 (actual was 14.9 μg ml^{-1}), 20 and 40 μg ml^{-1} cilostazol, and 100, 200, 600, 1000, 1500 (actual was 1491 μg ml^{-1}), 2000 and 4000 ng ml^{-1} of OPC-13015, OPC-13213, OPC-13217 and OPC-13326 were obtained by proportionally diluting the stock standard solutions with pre-screened blank microsomal incubation mixtures.

2.5. Sample handling

The microsomal incubation samples were provided by the Analytical Core Laboratory, Department of Clinical Pharmacology, Georgetown University Medical Center, Washington DC, USA. In general, liver microsomal incubation mixtures contain microsomal protein in sodium phosphate buffer and NADPH generating system, which is a mixture of β-NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase and magnesium chloride. In addition, appropriate amounts of the mentioned analytes and inhibitor probes were added to the incubation mixture to achieve the desired concentrations. Samples (500 μl) were precisely aliquoted and mixed with 50 μl acetonitrile in order to stop the metabolic reaction and precipitate the protein, and stored at −20°C until analysis. The calibration standards and quality assurance pools were prepared at the beginning of the study and stored along with the unknown study samples at −20°C until analysis.

2.6. Sample pre-treatment procedure

Samples (500 μl) of unknown microsomal incubation samples, calibration standards and quality assurance samples were pipetted into polypropylene micro test tubes. To each tube, 50 μl acetonitrile and then 100 μl of working internal standard solution were added. Acetonitrile was added to deactivate the proteins and to stop the metabolic reaction. The samples were vortexed for about 10–15 s and then microcentrifuged at a setting of 14000 rpm for 5 min. The supernatant (250 μl) was transferred into labeled Shimadzu autosampler vials. The samples were stored at 4°C until analysis and 100 μl were injected onto the HPLC system for analysis.

2.7. Chromatographic conditions

Analysis was performed by reverse-phase HPLC on a TSK-GEL ODS-80TM column (150×4.6 mm i.d.,5 μm) and a Cosmosil C18 column (150×4.6 mm i.d.,5 μm) in tandem. Mobile phase consists of the mobile phase A: 8% acetonitrile in 100 mM ammonium acetate buffer (pH 6.5), and the mobile phase B: 60% acetonitrile/100 mM ammonium acetate buffer (pH 6.5). An 80 min stepwise gradient program at a flow rate of 1.0 ml min^{-1} was used to mix different percentages of acetonitrile (Table 1), and was used to separate cilostazol, its metabolites and the internal standard from each other.

2.8. Calibration and calculation

After HPLC analysis of each batch, a standard calibration curve was plotted and the following regression parameters were calculated using the standard algorithms for weighted $(1/x)$, linear least-square regression: the slope $(1/a)$; the intercept $(-b/a)$; and the correlation coefficient. In addition, concentrations of the analytical standard solution were back-calculated using the same regression parameters to measure residuals and standard errors to validate the accuracy of the method. Quality assurance samples were prepared by a quality assurance officer and analyzed in a blinded manner. All the data was acquired using AS400, a Kansas City Analytical Services (KCAS) in-house data acquisition program [8].

Table 1
Mobile phase gradient program for chromatography

Time (min)	% Acetonitrile[a]
0.01	19.44
20.0	19.44
40.0	26.20
41.4	26.20
41.5	44.40
43.5	19.44
46.5	19.44
46.6	39.20
70.0	39.20
70.1	60.00
74.9	60.00
75.0	19.44
80.0	19.44

[a] A combination of 8% acetonitrile in ammonium acetate buffer (pH 6.5) and 60% acetonitrile in ammonium acetate buffer (pH 6.5) was used.

2.9. Assessment of validation

To validate the assay of cilostazol and the four primary metabolites in microsomal incubation samples, three calibration curves with standards in duplicate were analyzed in three different batches to complete the method validation. In every batch, quality assurance standards (QA standards) were prepared by spiking different amounts of cilostazol and the four metabolites into pre-screened blank microsomal incubation solutions at the following levels of sensitivity (lower limit of quantitation, LLOQ): low, medium and high concentrations. In every batch, the standard curve and the QA standards were evaluated to assess intra-batch and inter-batch reproducibility, accuracy and precision, respectively. The freeze/thaw cycle stability for the analytes was not assessed, as all the samples were extracted at one time for analysis. All the analytes were tested for stability in the autosampler at room temperature and found to be stable for at least 17 h.

3. Results

3.1. Specificity

Cilostazol, OPC-13015, OPC-13213, OPC-13217, OPC-13326 and the internal standard OPC-3930 were baseline separated from each other, with the resolution being 2.43–17.59 min. The blank microsomal incubation solution not spiked with any analytes showed no peaks which would interfere with either cilostazol, the four metabolites or the internal standard OPC-3930. The chromatographic separation for OPC-13213, OPC-13217, OPC-13326, OPC-13015 and cilostazol was accomplished with an efficiency of about 54000, 65000, 156000, 171000 and 112000 theoretical plates, respectively. The corresponding efficiency was about 166000 theoretical plates for

Table 2
Back-calculated concentrations of cilostazol and metabolites in microsomal incubation mixtures

	Concentrations (ng/ml)						
	1000[a]	2000	6000	10000	14909	20000	40000
Cilostazol	994 ± 5[c]	1976 ± 6	6023 ± 127	10309 ± 438	14778 ± 411	19769 ± 329	N/A[d]
	100[b]	200	600	1000	1491	2000	4000
OPC-13213	96 ± 8	200 ± 9	611 ± 16	1031 ± 40	1528 ± 48	1961 ± 5	3966 ± 6
OPC-13217	94 ± 6	199 ± 8	617 ± 14	1042 ± 40	1535 ± 44	1983 ± 4	3915 ± 5
OPC-13326	92 ± 4	199 ± 6	611 ± 18	1060 ± 19	1535 ± 34	2027 ± 64	3851 ± 4
OPC-13015	98 ± 5	199 ± 5	606 ± 23	1037 ± 24	1498 ± 29	1955 ± 39	N/A[d]

[a] Nominal levels of cilostazol (ng ml^{-1}).
[b] Nominal levels of metabolites (ng ml^{-1}).
[c] Data are presented as mean ± standard deviation; $n = 6$ for all analytes.
[d] N/A, data are not presented as levels of analytes outside the validated linear ranges.

OPC-3930. Fig. 2(D) shows a typical chromatogram in which all the analytes of interest were all resolved at baseline. Fig. 2(A) shows a typical chromatogram for a blank microsomal incubation sample with no analytes. The retention times were about 37, 41, 55, 57, 62 and 67 min for OPC-13213, OPC-13217, OPC-13326, OPC-3930, OPC-13015 and cilostazol, respectively. To ensure the specificity of the chromatogram, five metabolic inhibitors (i.e. quinidine sulfate, diethyl dithiocarbamate, omeprazole, ketoconazole and furafylline) were tested, showing no interfering peaks at the retention times of cilostazol, its metabolites and the internal standard. No significant problems such as back pressure, shift in retention times and interferences were observed with both columns being used continuously for at least 500 samples.

3.2. Relative sensitivity

The lower limit of quantitation (LLOQ) was set at 100 ng ml^{-1} for the metabolites and at 1000 ng ml^{-1} for cilostazol. Fig. 2(B) presents a typical chromatogram of a spiked microsomal incubation sample containing 100 ng ml^{-1} metabolites and 1000 ng ml^{-1} cilostazol. The peaks for all analytes were at least 3 times the background noise. Tables 4–8 present the performance of statistics for 12 spiked QA samples at this lower limit, which were assayed as unknowns on three occasions. The overall mean relative recoveries were 86.6, 84.3, 82.8, 81.2 and 88.2%, with the coefficient of variation (CV) being 5.9, 6.4, 3.9, 7.7 and 4.2% for OPC-13213, OPC-13217, OPC-13326, OPC-13015 and cilostazol, respectively.

3.3. Linearity

Linearity was established in the range 100–4000 ng ml^{-1} for OPC-13213, OPC-13217 and OPC-13326, 100–2000 ng ml^{-1} for OPC-13015, and 1000–20000 ng ml^{-1} for cilostazol, respectively, as shown in Tables 2 and 3. All three curves had correlation coefficients greater than or equal to 0.991. The linearity range was decided to be ideal to support the in vitro metabolism studies of cilostazol that are in progress; however, sensitivity at the lower end is possible but not explored. These data clearly demonstrated the

Table 3
Regression parameters of cilostazol and metabolites in microsomal incubation mixtures

Analyte	Slope[a]	Intercept[a]	Correlation coefficient[a]
Cilostazol	1898.32 ± 2.0	−65.58 ± 43.9	0.998
OPC-13213	1677.24 ± 8.6	−16.31 ± 1.4	0.998
OPC-13217	1579.88 ± 7.5	−19.56 ± 1.9	0.998
OPC-13326	977.73 ± 1.8	−28.60 ± 5.7	0.998
OPC-13015	2541.55 ± 0.5	−9.070 ± 7.9	0.999

[a] Mean ± SD.

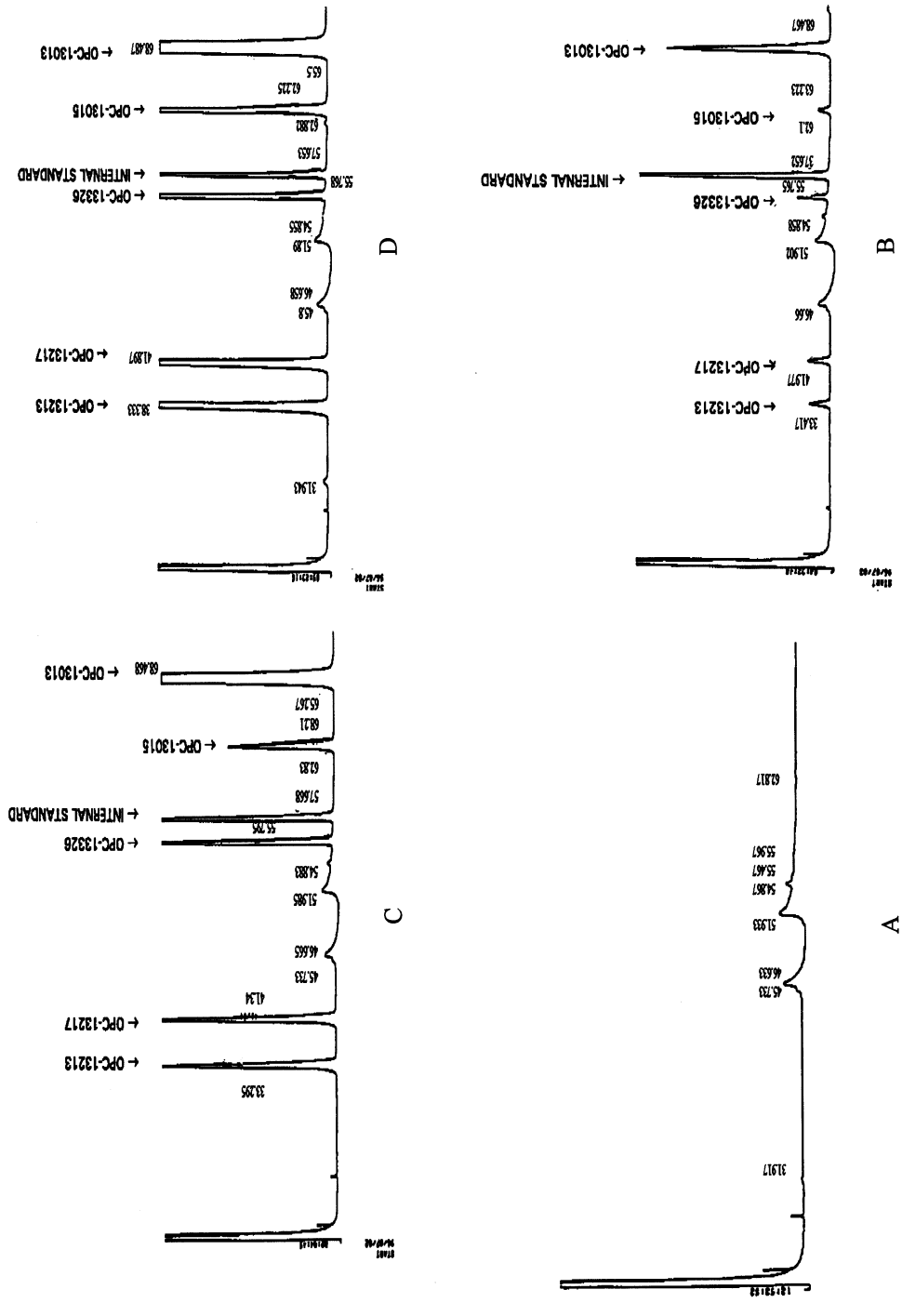

Fig. 2. Representative chromatograms of cilostazol and its metabolites in microsomal incubation mixtures: (A) blank microsomal incubation mixture; (B) microsomal incubation mixture containing LLOQ of the analytes; (C) microsomal incubation mixture containing 500 ng ml^{-1} cilostazol and 500 ng ml^{-1} of metabolites of the analytes; (D) microsomal incubation mixture containing ULOQ of the analytes.

Table 4
Intra- and inter- day accuracy and precision of microsomal quality control standards

Batch	*n*	Cilostazol			
		Theoretical (ng ml^{-1})	Observed (ng ml^{-1})	CV (%)	RE (%)
1	4	18182	17379	1.2	−4.4
	4	1000	875	2.5	−12.6
	4	2727	2512	2.3	−7.9
2	3[a]	18182	16148	8.3	−11.2
	4	1000	910	5.9	−9.0
	4	2727	2644	6.0	−3.0
3	4	18182	17973	1.3	−1.1
	4	1000	862	0.5	−13.8
	4	2727	2564	1.4	−6.0
Overall	11[a]	18182	17260	5.7	−5.1
	12	1000	882	4.2	−11.8
	12	2727	2573	4.1	−5.6

CV, coefficient of variation; RE, relative error.
[a] One value did not meet acceptance criteria.

linearity of the method and the reproducibility of the calibration standard curves.

3.4. Inter-batch accuracy and precision

Quality assurance pools containing cilostazol and metabolites were prepared and analyzed in blinded fashion as a form of internal validation. Tables 4–8 present the results of these assays, for which the relative recoveries of cilostazol and four metabolites ranged from 75.6 to 103.4% for four different concentrations, with the CV being 0.5–10.0%.

3.5. Intra-batch accuracy and precision

Similarly to the inter-batch accuracy and precision, quality assurance pools containing cilostazol and its metabolites were analyzed in a blinded manner as a form of internal validation. Tables 4–8 present the results of these assays, for which the relative recoveries of cilostazol and four metabolites ranged from 81.2 to 101.0% for four different concentrations, with the CV being 2.8–7.7%.

3.6. Stability

During validation of the method, freshly prepared solutions for both system validation solution and calibration standards showed no evidence of deterioration for either cilostazol, metabolites or the internal standard OPC-3930. The freeze/thaw stability for the analytes of interest was not determined, as limited volumes of study samples were expected from the in vitro metabolism studies requiring a one-time sample processing and analysis.

3.7. Analysis of microsomal incubation samples

Microsomal incubation samples obtained from a non-clinical study [9] were analyzed as outlined above. Fig. 3 presents the time course of metabolites formation when human liver microsomes were incubated with 25 μM cilostazol. A set of quality control standards was analyzed along with the microsomal samples, for which the relative recoveries of cilostazol and four metabolites ranged from 88.5 to 107.1% for four different concentrations, with the CV being 2.3–13.1%.

Table 5
Intra- and inter- day accuracy and precision of microsomal quality control standards

Batch	*n*	OPC-13213			
		Theoretical (ng ml^{-1})	Observed (ng ml^{-1})	CV (%)	RE (%)
1	4	1818	1854	2.4	1.9
	4	100	85	6.0	−15.3
	4	273	260	3.2	−4.6
	4	3636	3623	7.6	−0.4
2	3[a]	1818	1755	0.7	−3.5
	4	100	88	8.2	−12.1
	4	273	280	7.7	2.7
	4	3636	3398	2.5	−6.6
3	4	1818	1815	1.6	−0.2
	4	100	87	4.0	−12.9
	4	273	256	3.1	−6.1
	4	3636	3484	3.2	−4.2
Overall	11[a]	1818	1813	2.8	−0.3
	12	100	87	5.9	−13.4
	12	273	266	6.3	−2.7
	12	3636	3502	5.4	−3.7

CV, coefficient of variation; RE, relative error.
[a] One value did not meet acceptance criteria.

4. Discussion

The results of nonclinical and clinical studies [3,5] indicate that cilostazol is extensively metabolized. Several metabolites are pharmacologically active and found to be circulating in human plasma. Therefore, a reliable assay for cilostazol and its major metabolites was needed. An HPLC method was developed and validated for this purpose and to support a series of in vitro metabolism studies, such as: (1) the ability of cilostazol to inhibit cytochrome *P*-450 isoforms in human liver microsomes; (2) for investigation of human cytochrome *P*-450 isoform specific inhibitors to affect the metabolism of cilostazol in human liver microsomes, and also for investigation in drug–drug interaction studies. Among the 11 potential metabolites, OPC-13213 and OPC-13015 were chosen to be the analytes of interest, as they are the major circulating metabolites in human plasma after oral administration of cilostazol. OPC-13326 is an important metabolite that acts as an intermediary in the formation of OPC-13015, whilst OPC-13217 is a *cis*-isomer of OPC-13213; hence, they were also chosen to be analytes of interest, as they were expected to be generated in in vitro experiments. To accommodate added concentrations of cilostazol and expected concentrations of all the metabolites, this HPLC method was validated in the linearity range 1000–20000 ng ml^{-1} for cilostazol, 100–2000 ng ml^{-1} for OPC-13015, and 100–4000 ng ml^{-1} for OPC-13213, OPC-13217 and OPC-13326. The sensitivity of this HPLC method can be extended up to 20 ng ml^{-1}; however, this sensitivity was not needed for the current work. If a sensitivity of 20 ng ml^{-1} is needed, a split standard curve may be necessary to achieve a linear range of 20 ng ml^{-1} to the upper limit of quantitation. To ensure the specificity of the method, a 70-min gradient elution was established (Fig. 2(A)–(D)). Within this 70-min period, all the metabolites, cilostazol, and the internal standard OPC-3930 were clearly separated from each other.

The percent relative errors (% RE) of quality assurance (QA) pools at the level of 100 ng ml^{-1} for metabolites and 1000 ng ml^{-1} for cilostazol were consistently about −9 to −24% (Tables

Table 6
Intra- and inter- day accuracy and precision of microsomal quality control standards

Batch	*n*	OPC-13217			
		Theoretical (ng ml^{-1})	Observed (ng ml^{-1})	CV (%)	RE (%)
1	4	1818	1878	2.6	3.3
	4	100	82	5.5	−18.2
	4	273	259	2.5	−4.9
	4	3636	3415	7.9	−6.1
2	3[a]	1818	1779	1.2	−2.2
	4	100	89	4.6	−10.6
	4	273	282	8.8	3.4
	4	3636	3321	6.2	−8.7
3	4	1818	1835	1.7	0.9
	4	100	82	4.8	−18.4
	4	273	257	3.5	−5.7
	4	3636	3421	5.0	−5.9
Overall	11[a]	1818	1836	2.9	1.0
	12	100	84	6.4	−15.7
	12	273	266	6.9	−2.4
	12	3636	3386	6.1	−6.9

CV, coefficient of variation; RE, relative error.
[a] One value did not meet acceptance criteria.

4–8). This may be due to an error in the preparation of the QA pool as the % CV values of this pool were relatively small and only ranged from 0.5 to 8.6%. This speculative conclusion was reached as the % CV values of calibration standards at this level were very small ($\leq 7.8\%$), with the relative standard deviation also being less than 7.9% (Tables 2 and 3). The overall relative recoveries of this QA pool ranged from 81.2 to 88.2% and were within the 80–120% range suggested in the ICH guidelines [4]. The relative errors of all other QA pools were relatively small ($< 11.3\%$), which demonstrates the accuracy and precision of the analytical procedures.

Several columns with different packing materials and different lengths were evaluated for chromatography, with the combination of TSK-GEL and Cosmosil columns being found to provide the best resolution among the cilostazol, its metabolites, the internal standard and the endogenous background of human liver microsomal incubation samples. Although 80 min chromatography per sample was time consuming, it was necessary to ensure the quality of the chromatography, specificity and selectivity for the analytes of interest.

5. Conclusions

A reverse-phase HPLC method with ultraviolet detection was developed and validated for the quantitation of cilostazol and four of its principle metabolites (OPC-13015, OPC-13213, OPC-13217 and OPC-13326) in microsomal incubation mixtures. The results of the method demonstrated an acceptable resolution and specificity for the analytes of interest, whilst the constituents of the microsomal incubation mixtures and the general metabolic inhibitor probes (i.e. quinidine, diethyl dithiocarbamate, furafylline, omeprazole, and ketoconazole) did not interfere with the assay. The method described allows a lower limit of quantitation (LLOQ) of 100 ng ml^{-1} for OPC-13213, OPC-13217, OPC-13326 and OPC-13015, and 1000 ng ml^{-1} for cilostazol in microsomal incubation samples. The respective LLOQs for cilosta-

Table 7
Intra- and inter- day accuracy and precision of microsomal quality control standards

Batch	*n*	OPC-13326			
		Theoretical (ng ml^{-1})	Observed (ng ml^{-1})	CV (%)	RE (%)
1	4	1818	1876	4.0	3.2
	4	100	85	3.8	−15.0
	4	273	255	4.2	−6.5
	4	3636	3296	6.4	−9.4
2	3[a]	1818	1787	7.2	−1.7
	4	100	82	4.2	−17.6
	4	273	281	10.0	3.1
	4	3636	3421	6.0	−5.7
3	4	1818	1833	1.9	0.8
	4	100	81	2.6	−19.0
	4	273	268	5.0	−1.9
	4	3636	3420	4.8	−6.0
Overall	11[a]	1818	1836	4.5	1.0
	12	100	83	3.9	−17.2
	12	273	268	7.6	−1.8
	12	3637	3382	5.5	−7.0

CV, coefficient of variation; RE, relative error.
[a] One value did not meet acceptance criteria.

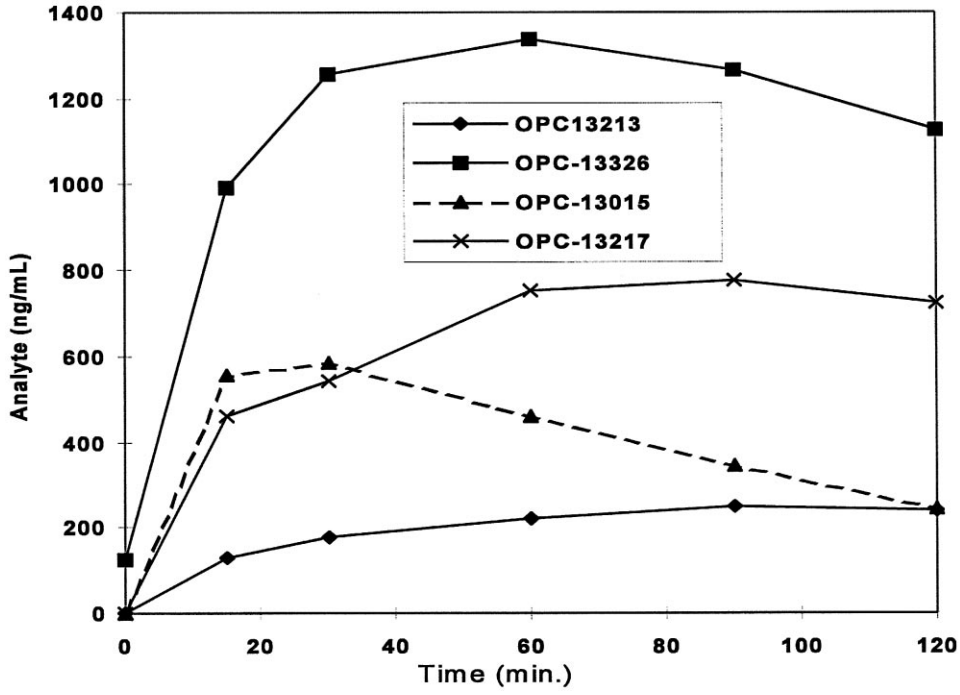

Fig. 3. Time course of the formation of cilostazol metabolites in human liver microsomal incubation mixtures.

zol and the metabolites were chosen to address the scope of the in vitro metabolism experiments. The sensitivity of this HPLC method can be extended up to 20 ng ml^{-1}. The method demonstrated acceptable performance and sensitivity at the lower limit of quantitation, linearity of analytical range, accuracy and precision of procedures, and stability of analytes. The method is currently used for the quantitative analysis of samples from the microsomal incubation samples in the studies investigating in vitro metabolism of cilostazol by human liver microsomes.

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