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Determination of cilostazol and its metabolites in human urine by high performance liquid chromatography

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Abstract

A high performance liquid chromatography (HPLC) method with ultraviolet detection for the simultaneous quantification of cilostazol, and its known metabolites in human urine was developed and validated. Cilostazol, its metabolites and the internal standard OPC-3930 (structural analogue of cilostazol) were extracted from human urine using liquid–liquid extraction with chloroform. The organic extract was then evaporated and the residue was reconstituted in 8% acetonitrile in ammonium acetate buffer (pH 6.5). The reconstituted solution was injected onto an HPLC system and was subjected to reverse-phase HPLC on a 5-μm ODS column. A gradient mobile phase with different percentages of acetonitrile in acetate buffer (pH 6.5) was used for the resolution of analytes. Cilostazol, its metabolites and the internal standard were well resolved at baseline with adequate resolution from constituents of human urine. The lower limit of quantification was 100 ng/ml for cilostazol and all metabolites. The method was validated for a linear range of 100–3000 ng/ml for all the metabolites and cilostazol. The overall accuracy (% relative recovery) of this method ranged from 86.1 to 116.8% for all the analytes with overall precision (%CV) being 0.8–19.7%. The long-term stability of clinical urine samples was established for at least 3 months at −20°C in a storage freezer. During validation, calibration curves had correlation coefficients greater than or equal to 0.995 for cilostazol and the seven tested metabolites. The method was successfully used for the analysis of cilostazol and its metabolites in urine samples from clinical studies, demonstrating the reliability and robustness of the method.

Keywords: Cilostazol; High performance liquid chromatography (HPLC); Organic extraction; Human urine; Metabolites

1. Introduction

Cilostazol [(6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H-quinolinone), OPC-13 013] is a novel quinolinone derivative synthesized by Otsuka Pharmaceutical Co., Ltd., Japan, and is currently approved and marketed

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for the treatment of intermittent claudication due to peripheral arterial disease [1]. Cilostazol has been reported to undergo extensive metabolism [2]. Previously available analytical methods used to assess plasma, urine and microsomal incubation samples [2–4] were not adequate for the clinical sample analysis because the available analytical methods either quantitated only cilostazol or were unable to separate endogenous interference from the analytes of interest. Hence, a reliable HPLC method was developed and validated for the simultaneous analysis of cilostazol and seven of its metabolites in human urine to support clinical studies designed to assess the renal elimination of cilostazol.

2. Experimental

2.1. Reagents

Cilostazol, its metabolites (OPC-13015, OPC-13213, OPC-13217, OPC-13366, OPC-13269, OPC-13326, OPC-13211, and OPC-1533) and the internal standard (OPC-3930) ($>99.0\%$), were obtained from Otsuka America Pharmaceutical Company, Inc. (Rockville, MD, USA, Tokushima, Japan). 1-Naphthoic acid was purchased from Aldrich Chemical Company, Milwaukee, WI 53201. All chemicals were of analytical reagent grade. HPLC-grade methanol, chloroform, ethyl acetate, and acetonitrile were purchased from Burdick & Jackson Laboratories (Muskegon, MI, USA), and analytical grade glacial acetic acid and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The structure of cilostazol, its major metabolites (OPC-13015 and OPC-13213), and the internal standard OPC-3930 are presented in Fig. 1.

2.2. Instrumentation

The HPLC system consisted of two Shimadzu Model LC-6A solvent delivery pumps, controlled by a Shimadzu SCL-6A system controller. Sam-

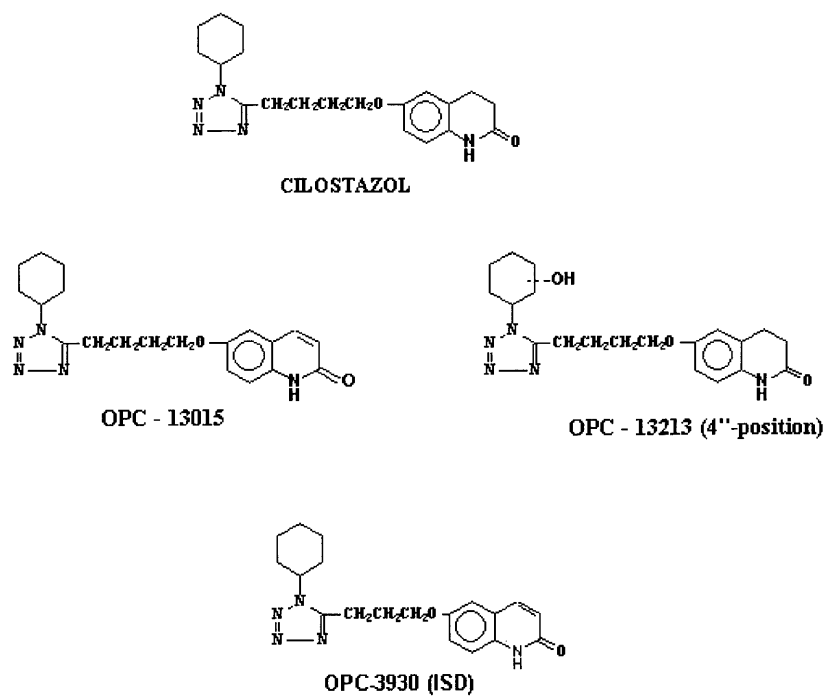

Fig. 1. Chemical structure of cilostazol, its primary metabolites and the internal standard.

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

Time (min)	%ACN/NH_4OAC buffer (100 mM, pH 6.5)
0.01	19.4
20.0	19.4
40.0	26.2
41.4	26.2
41.5	44.4
43.5	19.4
46.5	19.4
46.6	39.2
62.5	39.2
62.6	39.2
70.0	39.2
70.1	60.0
74.9	60.0
75.0	19.4
80.0	Stop

ples are injected by a Shimadzu SIL-6A autosampler, and resolved on a TSK-GEL ODS-80TM column (150×4.6 mm i.d., 5μ, TosoHaas Bioseparation Specialists, Inc. St. Louis, MO) preceded by a Cosmosil C18 column (150×4.6 mm i.d., 5μ, P.J. Cobert Associates, Inc. St. Louis, MO). The mobile phase was pumped at a flow rate of 1.0 ml in the gradient program described in Table 1. Eluents were monitored at 254 nm by a Shimadzu SPD-10A ultraviolet detector and peaks were integrated using a Shimadzu Model CR-3A integrator.

2.3. Analytical standards

Stock standard solutions consisting of cilostazol and its seven metabolites (50 μg/ml each) were prepared in methanol. The stock standard solution of the internal standard (50 μg/ml of OPC-3930) was also prepared in methanol. The working solutions of internal standard (10 μg/ml) were prepared by dilution with HPLC-grade deionized water. All stock solutions were stored at 4°C.

2.4. Calibration standards

Human urine calibration standards at 100, 200, 300, 500, 1000, 2000 and 3000 ng/ml of cilostazol and its metabolites were prepared by diluting the stock standard solutions with drug-free human urine. Quality control samples were prepared using drug-free urine samples by spiking appropriate amount of standard stock solutions. All calibration standards, quality control samples and clinical samples were stored at -20°C until analysis.

2.5. Sample extraction procedure

Human urine samples (0.5 ml), calibration standards and quality control samples were transferred into screw cap glass tubes, 100 μl of the working internal standard (10 μg/ml) was added and the contents were vortex-mixed. Five ml of chloroform was added to each tube and extracted for 20 min on a reciprocating shaker. The chloroform layer was separated into a clean glass screw cap tube and the urine layer was extracted with additional 5 ml of chloroform. The combined chloroform layer was evaporated to dryness under a stream of nitrogen at 37 ± 2°C. The residue was reconstituted in 1.0 ml of 8% acetonitrile in 100 mM ammonium acetate (pH 6.5). Two hundred microliters of the reconstituted solution were injected onto the HPLC system.

2.6. Chromatographic conditions for cilostazol and its metabolites

Analysis was performed by RP-HPLC using two analytical columns in sequence, a Cosmosil column (150×4.6 mm i.d., 5μ) followed by TSK-GEL ODS-80TM column (150×4.6 mm i.d., 5μ). An eighty min gradient program was used consisting of mobile phases; A (8% acetonitrile/100 mM ammonium acetate buffer; pH 6.5) and B (60% acetonitrile/100 mM ammonium acetate buffer; pH 6.5) at a flow rate of 1.0 ml/min. The gradient program for separating cilostazol and the seven metabolites is presented in Table 1.

2.7. Calibration curve and calculations

After a batch containing calibration standards, quality control samples and study samples were chromatographed and peak heights of all analytes and the internal standard were measured, peak height ratios were calculated and calibration curves were generated. The regression parameters of slope, intercept, and correlation coefficient were calculated by weighted $(1/x)$ linear regression. The concentrations of calibration standards were back-calculated using the following equation to measure residuals and S.E. to evaluate the validity of the method:

Analyte concentration = [(Analyte peak height /Internal standard peak height) − (Intercept)] /Slope

The analyte concentrations in quality control samples and samples were also computed using the above equation. All the data was acquired using an IBM AS400 system — a validated in-house data acquisition program.

2.8. Assessment of validation

To validate these assays for cilostazol and it metabolites in human urine samples, quality control samples were prepared by spiking different amounts of cilostazol and metabolites into drug-free blank human urine at three levels of sensitivity: (lower limit of quantification, LLOQ), medium and high concentrations. Four calibration curves with standards in duplicate were analyzed in four different batches to complete the method validation. In each batch, four quality control samples at each concentration were assayed to evaluate the intra- and inter-batch accuracy and precision, respectively. Due to the long chromatographic run time, about 68 h for a typical batch of 51 samples, it is difficult to evaluate intra-day variability hence only intra-batch and inter-batch precision and accuracy are reported. One quality control sample pool with a concentration at the middle concentration range was subjected to three freeze–thaw cycles, to evaluate the stability of the analyte under these conditions. Also, a quality control sample pool at the high concentration was diluted with blank human urine prior to extraction to evaluate the effect of dilution on precision and accuracy. To evaluate the long-term storage stability of analytes in human urine, samples from clinical studies were analyzed and then stored at −20°C for at least 3 months, then analyzed again with freshly prepared calibration standards.

3. Results

3.1. Specificity

Cilostazol, its metabolites and the internal standard were baseline separated from each other with resolution factors ranging of 1.45–9.58. The chromatographic separation of cilostazol, metabolites, and OPC-3930 was accomplished with an efficiency of approximately 122 054 and 184 134 theoretical plates for cilostazol and OPC-3930 respectively. Blank human urine not spiked with any analytes, showed no peaks that interfered with either cilostazol, or its metabolites OPC-13015, OPC-13213, OPC-13217, OPC-13366, OPC-13269, OPC-13326, OPC-13211 or the internal standard OPC-3930. Erythromycin, quinidine and its metabolites, metoprolol, hydroxymetoprolol, and warfarin did not interfere with this HPLC assay. Fig. 2A shows a typical chromatogram for a blank human urine sample with no analytes. Fig. 2B shows a typical chromatogram for a 3000 ng/ml calibration standard, demonstrating that cilostazol and all analytes of interest were baseline resolved. The chromatographic run time was about 75 min with the eluting sequence of analytes being OPC-13269 (34.9 min), OPC-13213 (40.9 min), OPC-13217 (43.8 min), OPC-13366 (45.4 min), OPC-13211 (55.6 min), OPC-13326 (58.6 min), OPC-3930 (internal standard, 60.4 min), OPC-13015 (66.0 min), and cilostazol (71.4 min).

3.2. Linearity

Weighted linear regression $(1/x)$ statistics are included in Table 2. Linear regression statistics

with and without a weighting factor ($1/x$ and $1/x^2$) were evaluated. The $1/x$ weighted function provide the best accuracy and precision for calibration standards for all analytes. Linearity, accu-

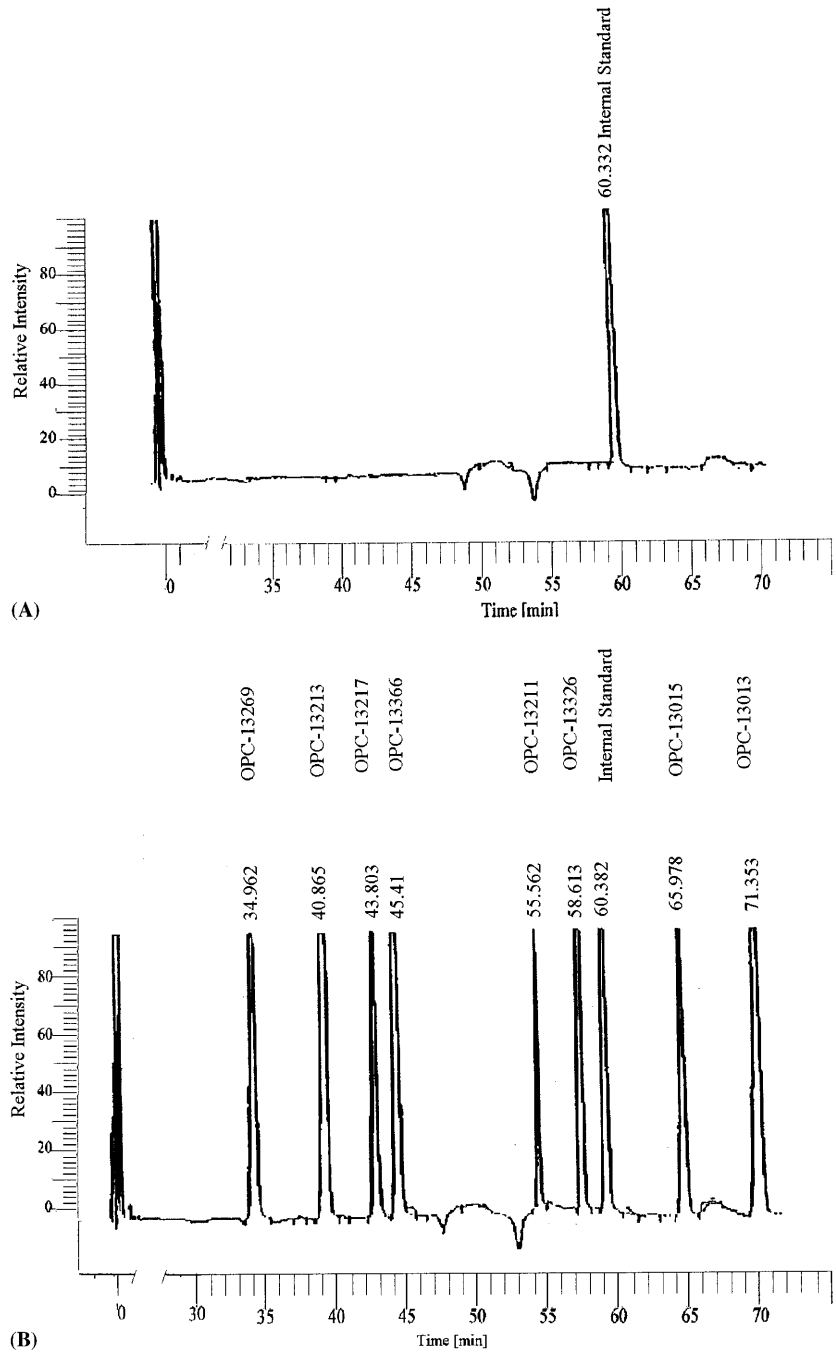

Fig. 2. (A) HPLC chromatogram of blank human urine. (B) HPLC chromatogram of cilostazol and its metabolites in human urine (3000 ng/ml). (C) HPLC chromatogram of cilostazol and metabolites in human urine (100 ng/ml).

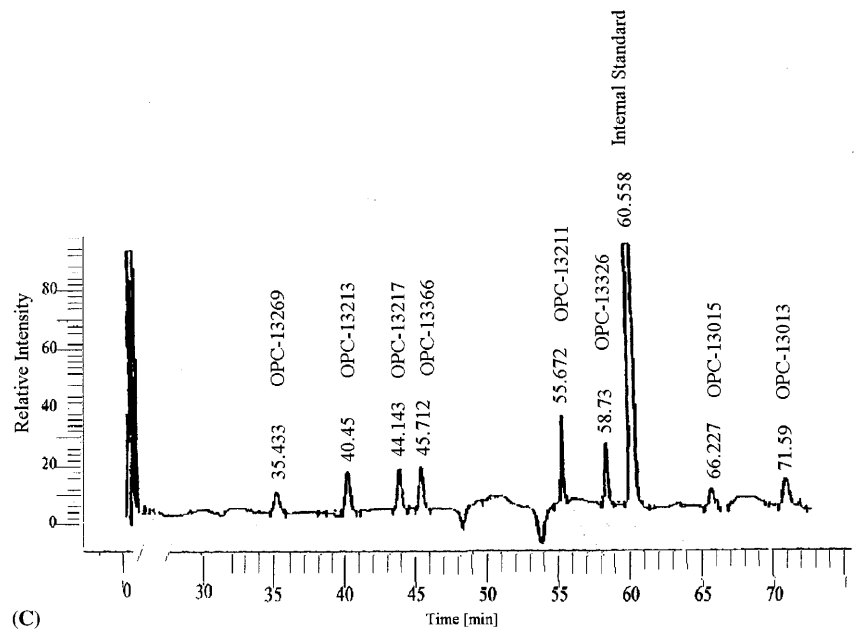

Fig. 2. (*Continued*)

racy, and precision were established in the range of 100–3000 ng/ml for cilostazol and all seven metabolites as shown in Tables 2 and 3. All four validation curves had correlation coefficients greater than or equal to 0.995 for cilostazol and the seven tested metabolites. These data clearly demonstrate the linearity of the methods and the reproducibility of the calibration standard curves.

3.3. Sensitivity

The LLOQ was set at 100 ng/ml for cilostazol and all seven metabolites using 0.5 ml of human urine. Fig. 2C presents a typical chromatogram of spiked human urine sample at the lower limit of quantitation (100 ng/ml of cilostazol and its metabolites). The peaks for all analytes are at least five to ten times the background noise. Table 4 presents the performance of quality control samples at this lower limit. The mean accuracy values were 103% (cilostazol), 101% (OPC-13269), 102% (OPC-13213), 101% (OPC-13217), 97.5% (OPC-13366), 99.7% (OPC-13211), 100% (OPC-13326), and 105% (OPC-13015), respectively. The corresponding% coefficients of variation (%CV) were 6.1% (cilostazol), 12.0% (OPC-13269), 11.0% (OPC-13213), 11.7% (OPC-13217), 11.4% (OPC-13366), 11.5% (OPC-13211), 10.5% (OPC-13326), and 13.9% for OPC-13015, respectively.

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

Table 4 presents the results of inter-batch precision and accuracy for which the relative accuracy for cilostazol and seven metabolites ranged from

Table 2
Regression statistical parameters for cilostazol and its metabolites in human urine (100–3000 ng/ml)

Analyte	Slope	Intercept	Correction coefficient
OPC-13013	4638 ± 268	-6.90 ± 3.8	0.999
OPC-13015	6551 ± 234	-4.31 ± 8.2	0.998
OPC-13213	2721 ± 702	6.06 ± 14.1	0.997
OPC-13217	2720 ± 681	3.72 ± 12.3	0.997
OPC-13366	2615 ± 668	0.47 ± 9.2	0.997
OPC-13211	1333 ± 152	-5.65 ± 18.1	0.998
OPC-13326	1963 ± 73.4	-1.00 ± 14.7	0.999
OPC-13269	5406 ± 1502	4.79 ± 5.0	0.996

Table 3
Back calculated concentrations of cilostazol and metabolites in calibration standards prepared in human urine (100–3000 ng/ml)

Analyte	100 ng/ml	200 ng/ml	300 ng/ml	500 ng/ml	1000 ng/ml	2000 ng/ml	3000 ng/ml
Cilostazol	99.3 ± 6.3[a]	204 ± 10.3	298 ± 13.4	493 ± 25.6	995 ± 50.0	2050 ± 42.2	2960 ± 46.5
OPC-13213	99.1 ± 13.9	225 ± 15.0	293 ± 18.4	467 ± 45.7	998 ± 90.3	2000 ± 88.9	3020 ± 136
OPC-13217	96.9 ± 12.4	222 ± 11.6	292 ± 22.2	472 ± 44.0	999 ± 73.3	2000 ± 103	3020 ± 141
OPC-13326	100 ± 3.8	210 ± 8.7	296 ± 21.1	477 ± 26.7	1010 ± 40.1	1990 ± 36.5	3020 ± 56.0
OPC-13015	96.8 ± 7.4	202 ± 10.8	299 ± 11.8	505 ± 35.4	1010 ± 28.0	2060 ± 84.8	2930 ± 66.7
OPC-13269	97.5 ± 17.0	217 ± 11.6	290 ± 22.9	469 ± 59.6	1010 ± 116.2	2020 ± 101	2980 ± 160
OPC-13211	99.3 ± 7.3	214 ± 7.3	298 ± 18.0	476 ± 45.1	986 ± 87.0	1980 ± 66.3	3050 ± 110
OPC-13366	95.4 ± 10.6	218 ± 11.3	303 ± 12.1	470 ± 40.7	1000 ± 89.0	2020 ± 112	3000.1 ± 160

[a] Data are presented as mean ± S.D.; $n = 4$ for all analytes.

93.6 to 105% of nominal values at three different concentrations with the precision (%CV) being 4.5–13.7%. Table 4 also presents the results of intra-batch precision and accuracy, for which the relative accuracy for cilostazol and seven metabolites ranged from 86.1 to 117% of nominal values at three different concentrations with the %CV being 0.8–19.7%.

3.5. Stability

Freshly prepared solutions such as system validation solution and working internal standard solution showed no evidence of degradation for either cilostazol, the seven metabolites or the internal standard OPC-3930. An approximately 4-h period was found to be necessary for sample processing and extraction of a typical batch of 51 samples. No significant degradation was observed for any analyte during the sample processing and extraction, including the dry down procedure. An analytical batch of 51 urine samples requires a 68-h run time. No stability problems were observed for samples stored in the refrigerator at 4°C with 20 samples or less placed in the autosampler at any given time during analysis (as a precaution). However the extracted samples were observed to be stable for at least 192 h if stored at 4°C and stable for up to 7 days at room temperature. Also, no significant degradation was observed for cilostazol or its metabolites in human urine samples when subjected to three freeze–thaw cycles. Urine samples collected from clinical studies of cilostazol were evaluated before and after storage at −20°C for stability and found to be stable for at least 3 months for cilostazol, OPC-13 015 and OPC-13 213. The other metabolites were not monitored over this storage period.

3.6. Dilution

To evaluate the influence of dilution procedure, samples with different concentrations were diluted two-, five-, ten- or 20-fold, with drug-free blank urine prior to extraction. The data indicated that the accuracy of cilostazol and its metabolites ranged from 86.3 to 103% with the %CV being 0.8–12.9%. The validation of 20-fold dilution procedures accommodates accurate and precise quantitation of cilostazol and its metabolites up to 60 000 ng/ml.

3.7. Absolute recovery

The absolute recoveries for cilostazol, its metabolites, and the internal standard were determined by comparing responses of the analytes in neat solution to responses after extraction from urine. The absolute recoveries ranged between 56% for cilostazol to 78% for OPC-13 269. The internal standard recovery was 58%.

4. Discussion

The results from non-clinical and clinical studies indicated that cilostazol is extensively metabolized [1]. Therefore, full understanding of the

Table 4
Precision accuracy, and sensitivity of cilostazol and its metabolites in human urine (quality control sample pools)

Analyte	Statistical variable	Inter-batch precision				Intra-batch precision ($n = 16$)			
		100 (ng/ml)	200 (ng/ml)	1000 (ng/ml)	2750 (ng/ml)	100 (ng/ml)	200 (ng/ml)	1000 (ng/ml)	2750 (ng/ml)
OPC-13013	% C.V.	2.3–7.6	2.1–6.1	2.2–5.3	3.2–4.1	6.1	5.4	5.1	4.4
	% Accuracy	96.3–105	100–106	98.3–107	101–104	103	103	102	101
OPC-13015	% C.V.	3.5–18.0	3.2–7.6	1.9–5.0	3.9–10.1	13.9	5.7	6.3	6.3
	% Accuracy	93.0–117	93.0–117	97.4–108	99.8–106	105	103	103	102
OPC-13213	% C.V.	4.4–17.5	2.7–13.7	7.8–12.7	4.50–14.8	11.0	11.4	11.0	9.8
	% Accuracy	94.1–107	89.7–105	88.2–101	91.7–104	102	97.7	93.6	95.9
OPC-13217	% C.V.	4.9–19.2	4.5–14.6	7.2–12.3	4.9–14.5	11.7	11.3	10.3	9.3
	% Accuracy	97.1–107	88.7–101	90.9–101	94.4–103	101	96.4	94.9	97.2
OPC-13366	% C.V.	6.2–19.7	3.8–14.1	7.7–11.8	6.4–14.8	11.4	11.2	10.2	9.4
	% Accuracy	95.3–100	87.0–102	89.5–100	93.7–103	97.5	97.5	94.9	97.3
OPC-13211	% C.V.	6.3–12.3	5.3–10.6	3.6–9.9	2.7–9.1	11.5	8.5	8.0	8.1
	% Accuracy	88.1–108	95.5–104	89.7–99.9	91.9–105	99.7	101	96.0	98.0
OPC-13326	% C.V.	4.6–10.3	0.8–6.0	3.1–6.5	4.5–5.3	10.5	5.1	5.3	5.6
	% Accuracy	87.7–106	95.8–105	95.3–102	96.2–104	100	101	98.9	99.9
OPC-13269	% C.V.	5.1–16.2	5.4–18.5	7.9–12.6	7.4–16.7	12.0	13.7	11.4	11.7
	% Accuracy	93.1–110	86.9–107	86.1–101	90.7–105	101	97.3	93.6	95.7

pharmacokinetics and metabolic profiles of cilostazol were essential and a reliable assay for cilostazol and its metabolites was needed. The initial goal was to validate a method to quantitate cilostazol and its potential metabolites with an analytical range of 100–3000 ng/ml. However, due to the lack of specificity and resolution for OPC-1533, only cilostazol and seven metabolites (OPC-13015, OPC-13213, OPC-13217, OPC-13366, OPC-13269, OPC-13326 and OPC-13211) could be successfully validated simultaneously. A separate HPLC method was developed and validated for OPC-1533 in human urine (not published). However, OPC-1533 was not detectable in human urine following dosing of clinical study participants with cilostazol.

The poor aqueous solubility of analytes (cilostazol, its metabolites and internal standard) in water was thought to be a potential problem. There was no evidence of precipitation observed when the working internal standards were prepared by diluting methanolic stock solutions with HPLC-grade water.

Chloroform was observed to effectively extract all the analytes of interest effectively from the human urine. Several HPLC columns with different packing materials and different column lengths were evaluated for chromatography. The combination of Cosmosil followed by TSK-GEL was found to perform the best resolutions among cilostazol, metabolites, the internal standard and the endogenous background of human urine. Combination of two HPLC columns was found necessary for resolving analytes of interest from the endogenous constituents of urine. The 80-min chromatographic run time per sample was time consuming, however, it was necessary to ensure the best specificity, resolution and selectivity for the analytes of interest. Within this 80 min, cilostazol, its seven metabolites, and the internal standard OPC-3930 were all separated from each other.

The overall relative errors of quality control samples ranged between 5.3 and 12.2%, and the overall accuracy ranged between 96.8 and 103%. Therefore adequate precision and accuracy was attained.

5. Conclusions

A reverse-phase HPLC method with ultraviolet detection at 254 nm was developed and validated for the quantification of cilostazol and seven metabolites (OPC-13 269, OPC-13 213, OPC-13 217, OPC-13 366, OPC-13 211, OPC-13 326 and OPC-13 015) in human urine. The results of the method validation demonstrated acceptable precision and accuracy with acceptable specificity and chromatographic resolution. In addition, all analytes were found acceptably stable under the conditions of storage, processing and analysis. The method achieved a LLOQ of 100 ng/ml in human urine samples. These methods were found to be suitable and robust for use in the assay of clinical samples.

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References

[1] S. Shintani, K. Watanabe, K. Kawamura, T. Mori, T. Tani, Y. Toba, H. Sasabe, N. Nakagiri, O. Hongoh, S. Fujita, T. Aogi, Y. Kido, M. Umezzato, M. Ishikawa, T. Hiyama, Arzneim-Forsch./Drug Res. 35 (II) (1985) 1163–1172.
[2] H. Akiyama, S. Kudo, S. Shimizu, Arzneim-Forsch./Drug Res. 35 (II) (1985) 1133–1140.
[3] H. Akiyama, S. Kudo, S. Shimizu, J. Chromatogr. Biomed. Appl. 338 (1985) 456–458.
[4] P.N.V. Tata, C.J. Fu, N.J. Browder, P.C. Chow, S.L. Bramer, J. Pharm. Biomed. Anal. 18 (1998) 441–451.