ELSEVIER

Journal of Chromatography B, 728 (1999) 251–262

JOURNAL OF
CHROMATOGRAPHY B

Simultaneous quantitative determination of cilostazol and its metabolites in human plasma by high-performance liquid chromatography

ChauHwei J. Fu[a], Prasad N.V. Tata[b], Keizo Okada[c], Hitoshi Akiyama[c], Steven L. Bramer[b,*]

[a]*Kansas City Analytical Services, 12700 Johnson Drive, Shawnee, KS 66216, USA*
[b]*Otsuka America Pharmaceutical, Inc., 2440 Research Boulevard, Rockville, MD 20850, USA*
[c]*Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima 771-01, Japan*

Received 17 August 1998; received in revised form 3 March 1999; accepted 3 March 1999

Abstract

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of cilostazol, a quinolinone derivative, and its known metabolites OPC-13015, OPC-13213, OPC-13217, OPC-13366, OPC-13269, OPC-13326 and OPC-13388 in human plasma was developed and validated. Cilostazol, its metabolites and two internal standards, OPC-3930 and OPC-13112, were extracted from human plasma by a combination of liquid–liquid and liquid–solid phase extractions, with combined organic solvents of *n*-butanol, methanol, chloroform, methyl-*tert*.-butyl ether, and a Sep-Pak silica column. The combined extract was then evaporated and the residue was reconstituted in ammonium acetate buffer (pH 6.5). The reconstituted solution was injected onto a HPLC system and was subjected to reversed-phase HPLC on a 5 μm ODS-80TM column to obtain quality chromatograph and good peak resolution. 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 two internal standards were well separated at baseline from each other with resolution factor being 74 and 138. This HPLC method was demonstrated to be specific for all analytes of interest with no significant interference from the endogenous substances of human plasma. The lower limit of quantitation was 20 ng/ml for cilostazol and all metabolites. The method was validated initially for an extended linear range of 20–600 ng/ml for all metabolites and cilostazol, and has been revised later for a linear range of 20–1200 ng/ml for cilostazol and two major and active metabolites OPC-13015 and OPC-13213. The overall accuracy (relative recovery) of this method was established to be 98.5% to 104.9% for analytes with overall precision (CV) being 1.5% to 9.0%. The long-term stability of clinical plasma samples was established for at least one year at −20°C. Two internal standards of OPC-3930 and OPC-13112 were evaluated and validated. However, the data indicated that there was no significant difference for all accuracy and precision obtained by using either OPC-3930 or OPC-13112. OPC-3930 was chosen as the internal standard for the analysis of plasma samples from clinical studies due to its shorter retention time. During the validation standard curves had correlation coefficients greater than or equal to 0.998 for cilostazol and the seven metabolites. These data clearly demonstrate the reliability and reproducibility of the method.
© 1999 Elsevier Science B.V. All rights reserved.

Keywords: Human plasma; Cilostazol; Metabolites

*Corresponding author. Tel.: +1-301-527-4760; fax: +1-301-721-7160.
E-mail address: steveb@mocr.oapi.com (S.L. Bramer)

0378-4347/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00104-8

1. Introduction

Cilostazol, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)-butoxy]-3,4-dihydro-2(1H-quinolinone), OPC-13013, Pletal; a novel quinolinone derivative synthesized by Otsuka Pharmaceutical, Japan, which is currently indicated for the treatment of intermittent claudication [1,2]. Cilostazol has been reported to undergo extensive metabolism [3]. Currently available analytical methods used to assess plasma, urine and microsomal incubation samples [3–5] could not be applied to the clinical investigation of cilostazol metabolism. These available analytical methods either quantitate only cilostazol or are unable to separate endogenous interference from the analytes of interest. Hence, initially a practical high-performance liquid chromatography (HPLC) method was developed and validated to quantitate cilostazol and seven metabolites simultaneously in human plasma to support the in vivo studies of cilostazol metabolism in humans. The structure of cilostazol, its metabolites and internal standards; OPC-3930 and OPC-13112 are presented in Fig. 1. This method was later simplified to quantitate cilostazol and two primary circulating and pharmacologically active metabolites, OPC-13015 {(6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)- butoxy]-2(1H-quinolinone)} and OPC-13213 {3,4-dihydro-6-[4-(1-(*trans*-4-hydroxycyclohexyl-1H-tetrazol-5-yl)butoxy]-2(1H-quinolinone)}.

2. Experimental

2.1. Reagents

Cilostazol (OPC-13013), its metabolites (OPC-13015, OPC-13213, OPC-13217, OPC-13371, OPC-13366, OPC-13269, OPC-13326, OPC-13388, OPC-13211 and OPC-1533) and the internal standards OPC-3930 and OPC-13112 (>99.0%), were obtained from Otsuka America Pharmaceutical (Rockville, MD, USA). All chemicals were of analytical reagent grade. HPLC-grade methanol, chloroform, methyl-*tert*.-butyl ether and acetonitrile were purchased from Burdick and Jackson Labs. (Muskegon, MI, USA), and analytical grade glacial acetic acid and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

The gradient HPLC system consisted of two Shimadzu Model LC-6A pumps, a Shimadzu SCL-6A system controller, a Shimadzu SIL-6A autosampler, a TSK-GEL ODS-80TM column (150×4.6 mm I.D., 5 μm, Tosohaas Bioseparation Specialists, St. Louis, MO, USA), a Shimadzu SPD-6A ultraviolet absorbance detector and a Shimazu Model CR-3A integrator. The wavelength of the detector

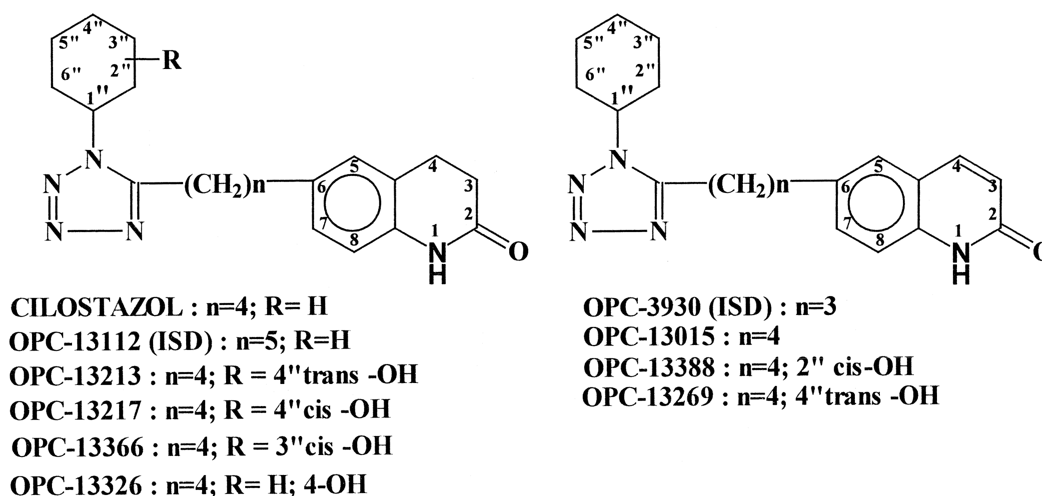

Fig. 1. Chemical structure of cilostazol, its metabolites and internal standards.

was set at 254 nm, which is the optimum ultraviolet absorbance for cilostazol.

2.3. Analytical standards

A stock standard solution consisting of cilostazol (50 μg/ml) and 10 metabolites (50 μg/ml) was prepared with methanol. The stock standard solution of the internal standards, OPC-3930 and OPC-13112 (25 μg/ml), was also prepared with methanol. The working solution of internal standard (2.5 μg/ml) was prepared by dilution with HPLC-grade deionized water (see Discussion for explanation). All stock solutions were stored at 4°C.

2.4. Calibration standards

Human plasma calibration standards of 20, 40, 100, 300 and 600 ng/ml of cilostazol and metabolites were obtained by diluting the stock standard solutions with drug-free human plasma using sodium heparin as the anticoagulant.

2.5. Handling of samples

For clinical samples, blood (10 ml) was drawn into vacutainer tubes containing sodium heparin as the anticoagulant. Immediately after collection, each blood specimen was centrifuged at 2500 rpm for 15 min at 4°C (refrigeration temperature) for plasma separation. The plasma was then transferred and frozen into labeled polypropylene tubes. The calibration standards and quality assurance pools were prepared at the beginning of the study and stored at −20°C until analysis.

2.6. Sample extraction procedures

Human plasma samples (1.0 ml), calibration standards and quality assurance samples, in which 100 μl of the working internal standard and 500 μl of 100 m*M* ammonium sulfate solution (pH 7.0) were added, were mixed with 10 ml of *n*-butanol–chloroform–methyl *tert*.-butyl ether (3:20:77, v/v/v). The samples were centrifuged at 2500 rpm and the aqueous and organic phases were separated at room temperature. The organic portion was mixed with 1.0 ml of 100 m*M* ammonium acetate (pH 6.5), dried down under nitrogen at 35°C to about 2.0 ml and saved as portion A. The aqueous portion was placed on the pre-conditioned Chem Elute CE 1003 and Sep-Pak silica columns and washed with 2×6.0 ml of chloroform by gravity. The analytes were then eluted with 5.0 ml of chloroform–methanol (50:50, v/v) by gravity. This eluent was then combined with the previously saved portion A of the initial organic extraction. The combined extract was dried down under nitrogen at 35°C to about 100 μl then was adjusted to about 1.0 ml with 100 m*M* ammonium acetate (pH=6.5) with 200 μl being injected into the HPLC system.

2.7. Chromatographic conditions

Analysis was performed by reversed-phase HPLC on a 5 μm TSK-GEL ODS-80TM column (150×4.6 mm I.D.). Mobile phase A was acetonitrile–100 m*M* ammonium acetate buffer (pH 6.5) (10:90, v/v) and mobile phase B was acetonitrile–100 m*M* ammonium acetate buffer (pH 6.5) (60:40, v/v). Flow-rate was 1.0 ml/min. The gradient programs used to mix different percentages of acetonitrile to separate cilostazol and the seven metabolites, and the short chromatography for cilostazol, OPC-13015 and OPC-13213 are presented in Table 1.

2.8. Calibration curve and calculations

After a batch of calibration standards, quality assurance samples and study samples were chromatographed and peak heights of both analyte and the internal standard were measured, a calibration curve was generated and 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 standard errors to evaluate the validity of the method:

$$\text{Analyte concentration} = (\text{slope}) \cdot \left(\frac{\text{analyte peak height}}{\text{internal standard peak height}} \right) + (\text{intercept})$$

Quality assurance samples were prepared by a

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

Time (min)	% ACN in NH_4OAc (100 m*M*; pH 6.5)
Long chromatography[a]	
0.0	10.00
30.0	25.00
37.5	31.85
60.0	45.00
60.5	60.00
61.5	10.00
75.0	10.00
Short chromatography[b]	
0.0	22.00
16.0	22.00
16.1	35.60
36.0	35.60
36.1	60.00
40.0	60.00
40.1	22.00
55.0	22.00

[a] Seven metabolites, 75-min gradient run.
[b] Two metabolites, 45-min gradient run.

quality assurance officer and analyzed in a blinded manner to the analysts (see Table 2). The results of these quality assurance samples were also computed using the above equation. All the data was acquired using IBM AS400 system, a validated Kansas City Analytical Services (KCAS) laboratory-made data acquisition program.

2.9. Assessment of validation

To evaluate the validity of this assay of cilostazol and seven metabolites in human plasma, calibration standards and quality assurance pools (QA pools) were prepared by spiking cilostazol and metabolites into drug-free blank human plasma at the levels of sensitivity (lower limit of quantitation, LLOQ), medium and high concentrations. Four calibration curves with standards in duplicate were analyzed in four different batches to complete the method validation. In a batch, each QA pool was assayed in sets of four to evaluate the accuracy and precision of intra- and inter-batches, respectively. Due to the long chromatographic time (about 66 h for a typical batch of 48 samples with 75-min run per sample or 72 samples with 55-min run per sample), it is difficult to evaluate and access intra-day variability. One QA pool with a concentration at the medium range was subjected to three freeze–thaw cycles to study the stability of all the analytes. Also, a QA pool at the high concentration was diluted with blank human plasma prior to extraction to evaluate the dilution procedures. To evaluate the long-term storage stability of analytes in human plasma, samples from clinical studies were analyzed first and then stored at −20°C for about 12.5 months, then analyzed again with freshly prepared calibration standards. Two internal standards of OPC-3930 and OPC-13112 were evaluated and validated. However, the data indicated that there was no significant difference for all accuracy and precision obtained by using either OPC-3930 or OPC-13112. Table 3 presents comparison between OPC-3930 and OPC-13112. OPC-3930 was chosen as the internal standard for the routine analysis of clinical samples because of its shorter retention time. Hence, only the data obtained using OPC-3930 is reported here.

3. Results

3.1. Specificity

Cilostazol, its metabolites and the internal standards were baseline separated from each other with resolution factors in the range of 74–138. The chromatographic separation of cilostazol, metabolites, OPC-3930 and OPC-13112 was accomplished with an efficiency of approximately 299,000 theoretical plates for cilostazol. Blank human plasma, not spiked with any analytes, showed no peaks that would significantly interfered with either cilostazol, OPC-13015, OPC-13213, OPC-13217, OPC-13366, OPC-13269, OPC-13326, OPC-13388 or the internal standards OPC-3930 and OPC-13112. Fig. 2a shows a typical chromatogram for a blank human plasma sample with no analytes. Fig. 2b shows a typical chromatogram where cilostazol and all analytes of interest are all baseline separated. The chromatographic retention times were between 7.5 and 58

Table 2
Precision, accuracy and sensitivity of cilostazol and its metabolites in human plasma[a] (quality assurance pools)

Analyte	Statistical variable	Inter-batch precision ($n=4$)			Intra-batch precision ($n=16$)		
		20 ng/ml	240 ng/ml	500 ng/ml	20 ng/ml	240 ng/ml	500 ng/ml
OPC-13013	Mean	18.50–21.64	237.60–247.64	518.85–533.68	20.01	242.10	524.71
	% CV	2.50–11.7	0.20–3.60	0.90–3.70	8.90	2.70	2.50
	% Recovery	92.50–108.20	99.00–103.20	103.80–106.70	100.10	100.90	104.90
OPC-13015	Mean	19.89–21.74	235.85–244.79	514.72–523.09	20.98	240.84	518.81
	% CV	2.20–8.50	1.60–4.50	1.10–1.80	6.90	2.80	1.50
	% Recovery	99.50–108.70	98.3–102.00	102.90–104.60	104.90	100.40	103.80
OPC-13213	Mean	19.62–20.50	238.97–253.06	482.49–528.18	20.09	245.00	502.31
	% CV	1.90–7.70	1.30–4.10	1.30–4.40	5.10	3.30	4.10
	% Recovery	98.10–102.50	99.60–105.40	96.50–105.60	100.50	102.10	101.10
OPC-13217	Mean	19.63–20.91	237.75–253.78	483.85–531.23	20.12	244.29	505.51
	% CV	2.20–6.10	1.50–4.00	0.40–4.30	4.60	3.50	4.10
	% Recovery	98.20–104.60	99.10–105.70	96.80–106.20	100.60	101.80	101.80
OPC-13366	Mean	19.33–21.13	238.04–255.03	505.38–544.92	20.37	245.44	520.53
	% CV	2.50–5.00	1.40–4.30	1.20–4.70	5.10	3.60	3.80
	% Recovery	96.70–105.70	99.20–106.30	101.10–109.00	101.90	102.30	104.10
OPC-13388	Mean	18.15–20.52	240.09–256.97	481.44–517.48	19.77	245.08	499.42
	% CV	2.80–9.80	1.40–2.50	0.70–3.70	7.50	3.40	3.20
	% Recovery	90.80–102.60	100.00–107.10	96.30–103.50	98.90	102.10	99.90
OPC-13326	Mean	17.36–21.48	241.80–255.23	513.05–542.86	20.10	245.48	523.78
	% CV	2.60–4.40	0.90–3.70	0.80–4.10	9.00	3.20	3.30
	% Recovery	86.8–107.40	100.80–106.30	102.60–108.60	100.50	102.30	104.80
OPC-13269	Mean	19.60–20.23	234.19–253.18	452.64–523.52	19.91	243.77	492.49
	% CV	2.60–7.20	1.30–4.00	1.80–9.10	5.10	4.00	7.00
	% Recovery	98.00–101.20	97.60–105.50	90.50–104.70	99.60	101.60	98.50

[a] Using OPC-3930 as internal standard. Quality assurance standards.

min. The eluting sequence of analytes was OPC-13269 (31.3 min), OPC-13213 (33.4 min), OPC-13217 (35.3 min), OPC-13366 (36.2 min), OPC-13388 (38.7 min), OPC-13326 (46.1 min), OPC-3930 (internal standard, 48.8 min), OPC-13015 (52.8 min), cilostazol (55.6 min) and OPC-13112 (internal standard, 58.0 min). For the modified short chromatography, the retention times of OPC-13213, OPC-3930 (internal standard), OPC-13015 and cilostazol were about 14.5, 27.4, 32.0 and 36.0 min, respectively. No significant alternations in pressure were observed with the HPLC analytical column being used continuously for at least 500 samples.

3.2. Linearity

Linearity was initially validated in the range of 20–600 ng/ml for cilostazol and all seven metabolites. The LLOQ of 20 ng/ml was based on the early pharmacokinetic data available from Japanese studies. This linearity was extended and validated later up to 1200 ng/ml for cilostazol and two major circulating and pharmacologically active metabolites, OPC-13015 and OPC-13213 as plasma levels of cilostazol were observed to be higher than 600 ng/ml in the clinical studies. All validation curves have correlation coefficients (r) are greater than or equal

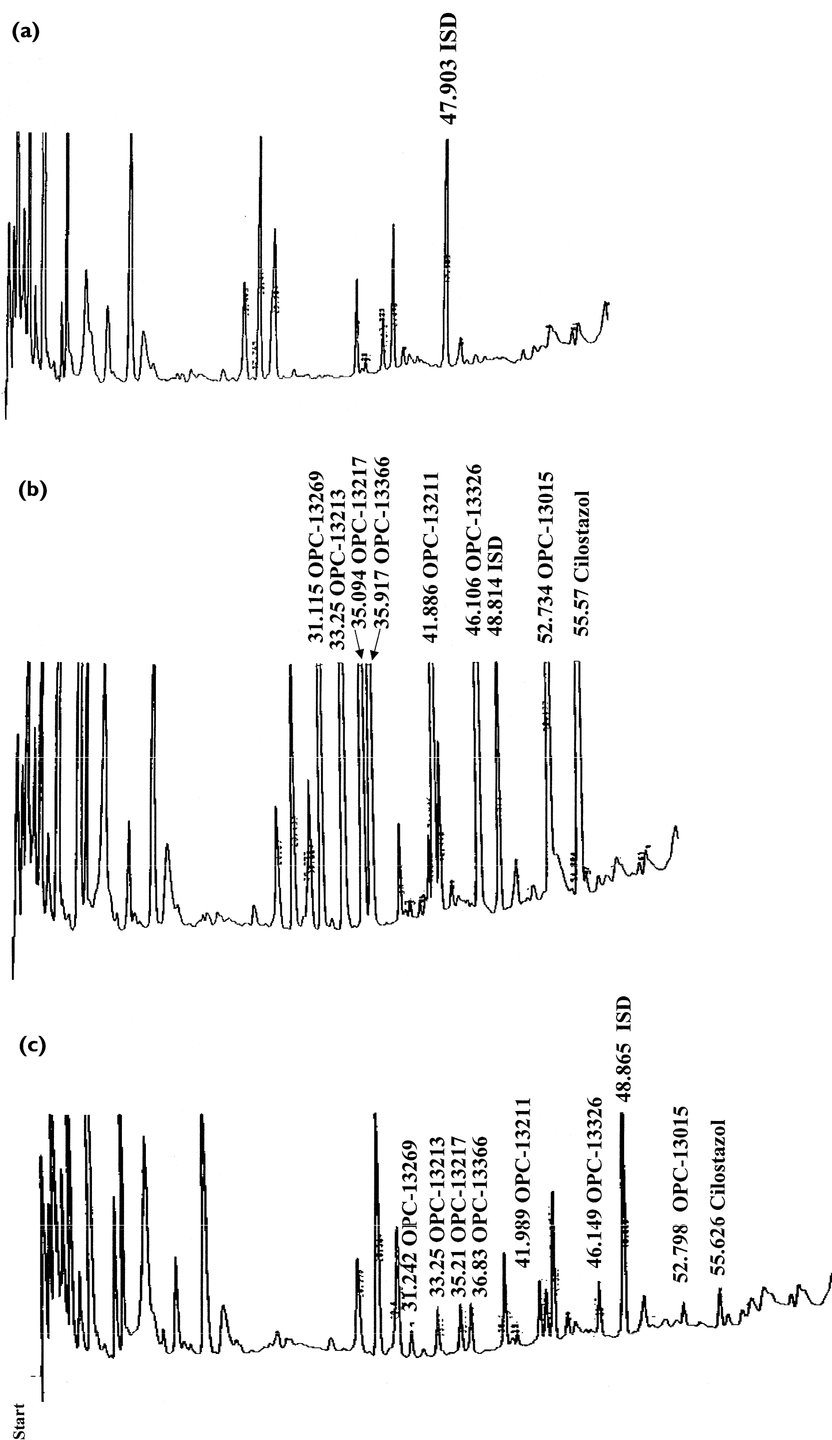

Fig. 2. (a) HPLC chromatogram of blank human plasma (long run). (b) HPLC chromatogram human plasma standard (1200 ng/ml) (long run). (c) HPLC chromatogram human plasma standard (20 ng/ml) (long run).

to 0.998 ($n=4$) for cilostazol and seven metabolites. These data clearly demonstrate the linearity of the method and the reproducibility of the calibration standard curves.

3.3. Sensitivity

The LLOQ was set at 20 ng/ml for cilostazol and all seven metabolites using 1.0 ml of human plasma. Fig. 2C presents a typical chromatogram of human plasma sample spiked with 20 ng/ml of cilostazol and metabolites. The peaks for all analytes are at least three-times the background noise. Table 3 presents the performance of QA standards at this lower limit, which were assayed as unknowns on four occasions. The mean relative recoveries were 100.1% (Cilostazol), 99.6% (OPC-13269), 100.5% (OPC-13213), 100.6% (OPC-13217), 101.9% (OPC-13366), 98.9% (OPC-13388), 100.5% (OPC-13326) and 104.9% (OPC-13015) with the coefficient of variation (CV) being 8.9% (Cilostazol), 5.1% (OPC-13269), 5.1% (OPC-13213), 4.6% (OPC-13217), 5.1% (OPC-13366), 7.5% (OPC-13388) and 9.0% (OPC-13326), respectively.

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

Analysis of quality assurance pools to which cilostazol and metabolites were prepared in a blinded

Table 3
Comparison OPC-3930 and OPC-13112 as internal standards – performance of 20 ng/ml QA standard

Analyte	Statistical variable	Among batch precision ($n=16$)	
		OPC-3930 as I.S.D.	OPC-13112 as I.S.D.
OPC-13013	Mean	20.01	19.58
	% CV	8.90	8.3
	% Recovery	100.10	97.9
OPC-13015	Mean	20.98	20.51
	% CV	6.90	6.4
	% Recovery	104.90	102.6
OPC-13213	Mean	20.09	19.83
	% CV	5.10	7.6
	% Recovery	100.50	99.2
OPC-13217	Mean	20.12	19.80
	% CV	4.60	8.8
	% Recovery	100.60	99.0
OPC-13366	Mean	20.37	20.05
	% CV	5.10	10.1
	% Recovery	101.90	100.3
OPC-13388	Mean	19.77	19.49
	% CV	7.50	7.3
	% Recovery	98.90	97.5
OPC-13326	Mean	20.10	19.81
	% CV	9.00	6.6
	% Recovery	100.50	99.1
OPC-13269	Mean	19.91	19.69
	% CV	5.10	8.2
	% Recovery	99.60	98.5

[a] CV=Coefficient of variation.

manner and were employed as a form of internal validation. Table 2 presents a summary of inter-batch precision and accuracy, for which the relative recoveries of cilostazol and seven metabolites ranged from 98.5 to 104.9% for three different concentrations with the CV being 1.5–9.0%. Table 2 also presents intra-batch precision and accuracy, for which the relative recoveries of cilostazol and seven metabolites ranged from 86.8 to 109.0% for three different concentrations with the CV being 0.2–11.7%.

3.5. Validation of cilostazol, OPC-13213 and OPC-13015 with short (45 min) chromatographic run time

The original chromatographic time was about 75 min per run, which is essential to separate cilostazol, seven metabolites and two internal standards from each other (Fig. 2a and b). A revised, 45 min run time chromatography (Fig. 3a and b) was established and validated for cilostazol and its two major circulating and pharmacologically active metabolites; OPC-13015 and OPC-13213. To validate this new chromatographic condition, plasma samples collected from patients in a clinical study were analyzed with both short and long chromatography. Less than 2.5% variation was observed in the mean results between the two methods for cilostazol and its two major metabolites (Table 4).

3.6. Stability

Freshly prepared solutions such as system validation solution and working internal standard solution showed no evidence of degradation for either cilostazol, or the seven metabolites, or the internal standards OPC-3930 or OPC-13112. An approximately 7-h period was found to be necessary for sample processing and extraction of a typical batch of 48–72 samples. However, no significant degradation could be observed for all analytes of interest during the sample process and extraction, which includes dry-down procedure with nitrogen at 35°C. An analytical batch requires (48 samples using the long chromatography or 72 samples using short chromatography) 66 h run time. No stability problems were observed if, samples were stored in the refrigerator at 4°C and only 20 samples or less are placed in the autosampler analysis. The extracted samples were observed to be stable at 4°C in the refrigerator for up to one week. Also, no significant degradation was observed for cilostazol and all seven metabolites in human plasma samples when subjected to three freeze–thaw cycles. Plasma samples collected from clinical studies of cilostazol were also analyzed and showed that cilostazol, OPC-13015 and OPC-13213 were stable if human plasma samples were stored at −20°C for up to 12.5 months. The stability of plasma samples stored at −70°C has not been evaluated.

3.7. Dilution

To evaluate the dilution procedures, samples with different volumes were diluted two- or 10-fold with drug-free blank plasma prior to extraction. The data indicated that the relative recoveries of cilostazol and seven metabolites ranged from 96.4% to 109.5% with the CV being 1.2–7.9%. The validation of 10-fold dilution procedures could extend the upper limit of quantitation for this analytical method to 12 000 ng/ml.

4. Discussion

The results from the non-clinical studies and clinical studies indicate that cilostazol is extensively metabolized [3,6]. Several metabolites are pharmacologically active. These studies also indicate that co-administration of other medications may change the metabolic profiles of cilostazol. Therefore, full understanding of the kinetic and metabolic profiles of cilostazol was essential and a reliable assay for cilostazol and its metabolites was needed. The initial goal was to validate a method to quantitate cilostazol and all ten potential metabolites with an analytical range of 20 ng/ml to 600 ng/ml. However, due to the lack of specificity and resolution for OPC-1533, OPC-13211 and OPC-13371, only cilostazol and seven metabolites (OPC-13015, OPC-13213, OPC-13217, OPC-13366, OPC-13269, OPC-13326 and OPC-13388) could be successfully validated. Among the 10 potential metabolites, OPC-13213 and OPC-13015, as well as cilostazol were later chosen to be

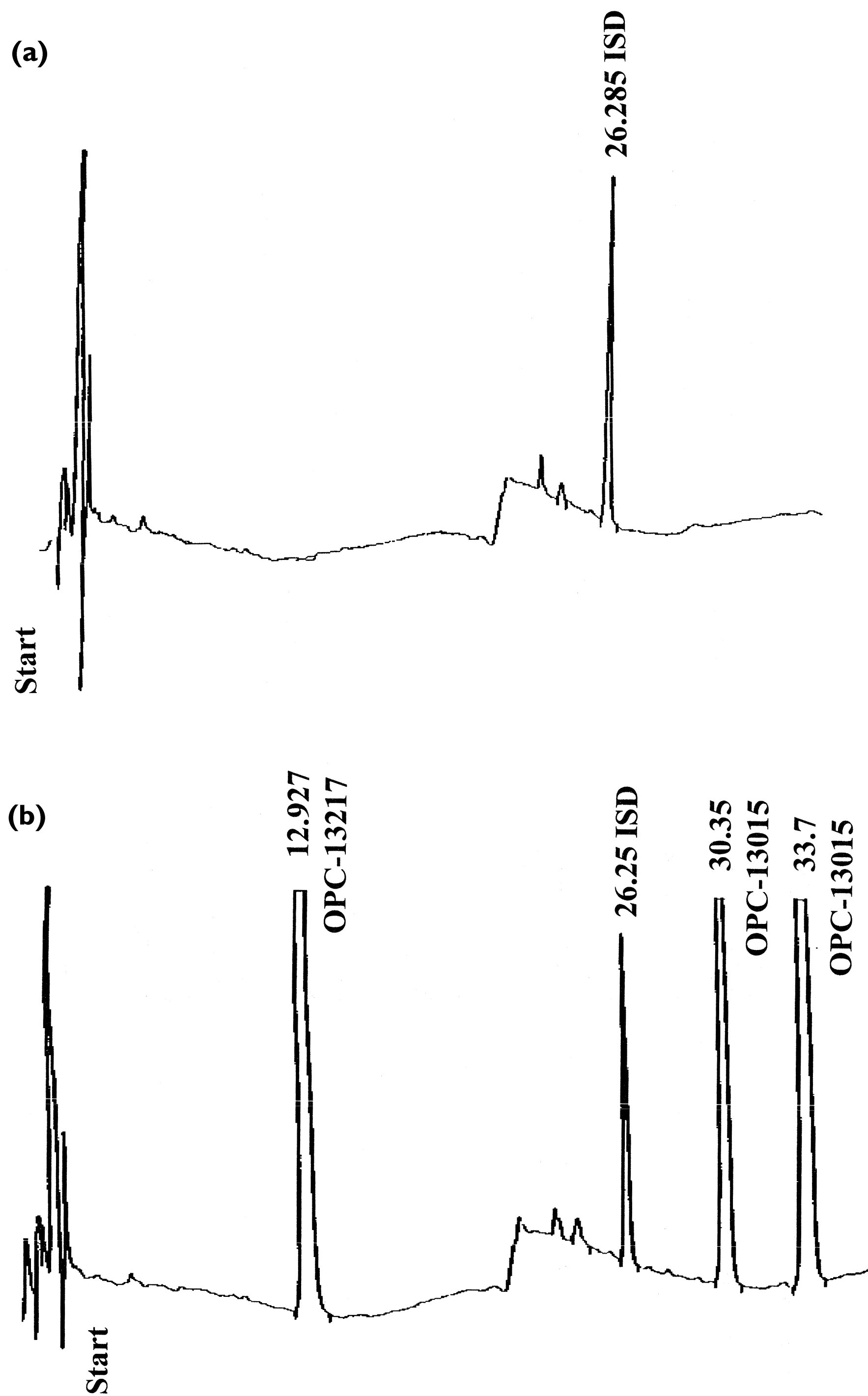

Fig. 3. (a) HPLC chromatogram of blank human plasma (short run). (b) HPLC chromatogram human plasma standard (1200 ng/ml) (short run).

Table 4
The comparison between long and short chromatography for cilostazol, OPC-13213 and OPC-13015

	Clinical samples		Quality assurance pools	
	Long chromatography	Short chromatography	Long chromatography	Short chromatography
Cilostazol	$n=33$ Mean = 100.0% [a]	$n=33$ Mean = 100.2% SD = 1.2 % CV = 1.2	$n=6$ Mean = 100.0% [a]	$n=6$ Mean = 100.6% SD = 3.1 % CV = 3.1
OPC-13213	$n=31$ Mean = 100.0% [a]	$n=31$ Mean = 102.4% SD = 8.0 % CV = 7.8	$n=6$ Mean = 100.0% [a]	$n=6$ Mean = 101.8% SD = 1.0 % CV = 1.0
OPC-13015	$n=32$ Mean = 100.0% [a]	$n=32$ Mean = 98.5% SD = 5.8 % CV = 5.9	$n=6$ Mean = 100.0% [a]	$n=6$ Mean = 99.4% SD = 4.0 % CV = 4.0

[a] % Ratio = (short chromatography/long chromatography) × 100%; SD = standard deviation; CV = coefficient of variation.

the analytes of interest as they are the major circulating metabolites in human plasma after oral administration of cilostazol. A short chromatography of 45-min gradient run was then established and validated for this purpose. The analytical range was extended to 1200 ng/ml as higher plasma concentrations were observed in human plasma after the initial method was validated. Two internal standards were initially evaluated for this method, respectively, for all analytes. The results obtained by using OPC-3930 as the internal standard were observed not to be significantly different from those using OPC-13112 as the internal standard (Table 3). OPC-3930 was chosen to be the internal standard because of its shorter retention time (~27.0 min).

The simplified liquid–liquid and liquid–solid phase extraction procedures have been evaluated, respectively, for sample purification before chromatography. However, the combination of both liquid–liquid and liquid–solid phase extractions was found to be advantageous in providing the selective extraction of the analytes from endogenous substances and improved recovery of all the analytes. For improving the recovery of analytes from plasma matrix we attempted 2×10 ml organic solvent mixture, this resulted in the extraction of endogenous material, hence the aqueous portion left after the initial organic extraction was subjected to solid-phase extraction. Extracts from initial organic extraction and elute from the solid-phase extraction were combined and the residue after evaporating the combined organic extracts was reconstituted in 0.1 *M* ammonium acetate buffer, and this process resulted in a cleaner chromatograms improved recovery and precision.

Several columns with different packing materials and different lengths have been evaluated for chromatography and the TSK-GEL ODS column was found to perform the best resolutions among cilostazol, metabolites, two internal standards and the endogenous background of human plasma. The 75-min chromatography per sample was time consuming however it was necessary to ensure the best specificity, resolution and selectivity for the analytes of interest. Within this 75 min, all 10 metabolites, cilostazol and the internal standards OPC-3930 or OPC-13112 were all separated from each other, although only seven metabolites were finally validated for human plasma.

Although the limited solubility of analytes (cilostazol, its metabolites and internal standards of OPC-3930 and OPC-13112) in water was thought to be a potential problem, but there was no evidence of precipitation observed when the working internal standards were prepared by diluting methanolic stock solutions with HPLC-grade water. Sodium heparin was chosen to be the anticoagulant for plasma collection during the method validation. However,

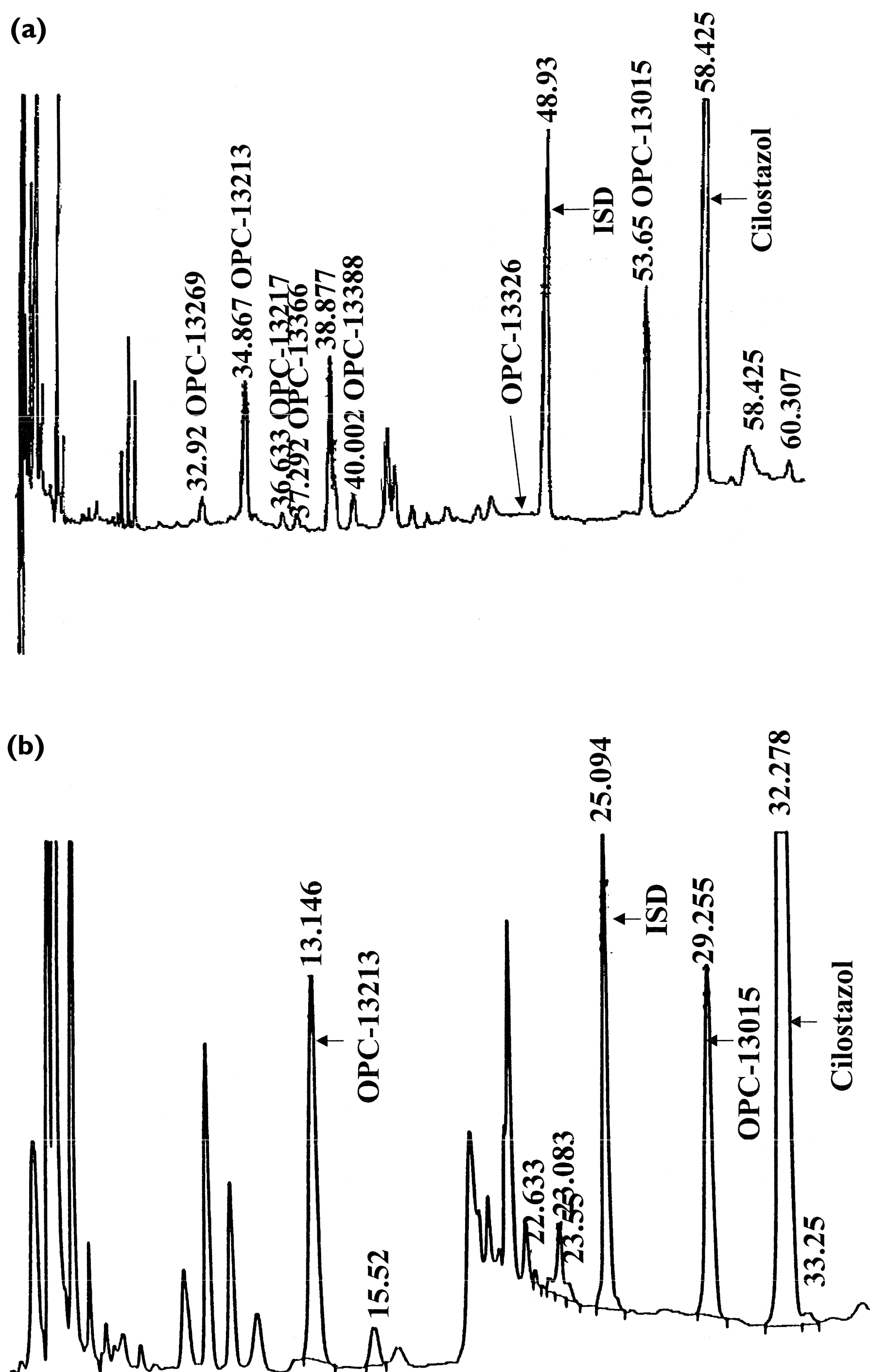

Fig. 4. (a) HPLC chromatogram of human plasma of a subject treated with cilostazol (long run). (b) HPLC chromatogram of human plasma of a subject treated with cilostazol (short run).

the use of potassium EDTA as an anticoagulant was observed and did not pose any problem.

The overall relative errors of quality assurance pools ranged from −1.5% to 4.9%, which were within the ±20% range generally accepted in the pharmaceutical industry. Therefore, these relative errors and precision demonstrate the quality of the chromatography and the accuracy and precision of the analytical procedures. Fig. 4a (long run) and 4b (short run) show representative HPLC chromatograms for the plasma samples collected from human subjects treated with cilostazol (100 mg bid p.o. dose). The two methods were suitable for quantitative assay of cilostazol and its metabolites in plasma samples from clinical studies.

5. Conclusions

A reversed-phase HPLC method with ultraviolet detection at 254 nm was developed and validated for the quantitation of cilostazol and seven metabolites (OPC-13269, OPC-13213, OPC-13217, OPC-13366, OPC-13388, OPC-13326 and OPC-13015) in human plasma. The results of the method validation demonstrate acceptable performance and high quality for the specificity and resolution of chromatography. The method described allows a LLOQ of 20 ng/ml in human plasma samples. The respective LLOQ for cilostazol and metabolites were chosen to address the scope of the human metabolism studies. The method also demonstrates acceptable performance for the linearity of analytical range, accuracy and precision of procedures, and stability of analytes.

Acknowledgements

The authors wish to acknowledge the support of Mr. Jerry Brisson of OAPI for his critical review of the manuscript and Ms. Marilyn Rogers of KCAS for her assistance during the finalization of the manuscript.

References

[1] S. Shintani, Y. Toba, S. Suzuki, S. Ninomiya, M. Umezato, T. Hiyama, Arzneim-Forsch./Drug Res. 35 (1985) 1157–1162.

[2] 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 (1985) 1163–1172.

[3] H. Akiyama, S. Kudo, S. Shimizu, Arzneim-Forsch./Drug Res. 35 (1985) 1133–1140.

[4] H. Akiyama, S. Kudo, S. Shimizu, J. Chromatogr. 338 (1985) 456–458.

[5] P.N.V. Tata, C.J. Fu, N.J. Browder, P.C. Chow, S.L. Bramer, J. Pharm. Biomed. Anal. (1998) in press.

[6] S.L. Bramer, P.N.V. Tata, Xenobiotica (1999) submitted for publication.