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Simultaneous determination of clofibrate and its active metabolite clofibric acid in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet absorbance detection

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) using ultraviolet (UV) absorbance detection method for simultaneous determination of clofibrate (**I**) and its major metabolite clofibric acid (**II**) in human plasma has been developed to support a clinical study. **I**, **II** and internal standard (I.S., **III**) are isolated from human plasma by 96-well solid-phase extraction (SPE) C_{18}·AR plate and quantified by direct injection of the SPE eluent onto the HPLC with UV detection wavelength at 230 nm. Two chromatographic methods, isocratic and step gradient, have been validated from 1.0 to 100.0 μg/ml and successfully applied to plasma sample analysis for a clinical study. The lower limit of quantitation (LLOQ) is 1.0 μg/ml for both **I** and **II** when 500 μl plasma sample is processed. Sample collection and preparation is conducted at 5 °C to minimize the hydrolysis of **I** to **II** in human plasma.

Keywords: Clofibrate; Clofibric acid

1. Introduction

Compound **I** was the first agent in the fibrate class of drugs that lowered the elevated serum lipids by acting as an agonist of the nuclear transcriptional regulator, the peroxisome proliferation activated receptor α (PPARα). However, recent studies have found that **I** increases the cholesterol content of bile and consequently the lithogenicity index in both patients [1,2] and healthy subjects [3,4]. In doing so, the potential risk of gallstone formation may increase. A clinical study was established to further determine the day-to-day variability of biliary lipid composition and to assess the change in cholesterol saturation of bile that was induced by **I** in healthy young males. To measure the exposure of **I** and its metabolite **II** in this study (Fig. 1), a reliable high-perfromance liquid chromatography (HPLC) method for simultaneous determination the concentrations of **I** and **II** in human plasma was essential.

The reported HPLC methods for simultaneous determination of **I** and **II** in plasma used liquid–liquid extraction (LLE) [5,6]. However, our investigations found that LLE was not an appropriate isolation procedure for **I** because of its low boiling point (BP: 148–150 °C): its volatility resulted in large losses during evaporation of the extraction solvent. The loss not only affected the LLOQ but also resulted in poor linearity and reproducibility of

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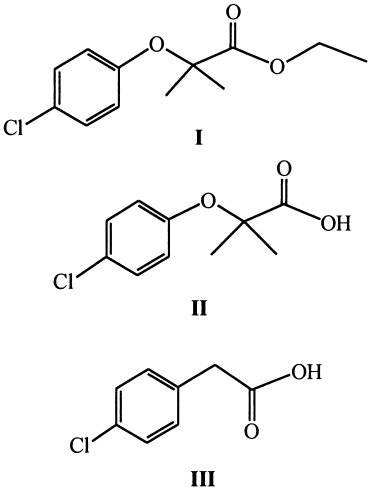

Fig. 1. Chemical structures of **I**, **II** and **III**.

the calibration curve. Since solid-phase extraction (SPE) eliminated the drug loss during the evaporation step, an isocratic reversed-phase HPLC method coupled with SPE was developed and validated. Under these assay conditions, matrix stability, autosampler stability and freeze–thaw stability were assessed and a proper pH to improve the stability of **I** in plasma was determined. The assay was modified to a step-gradient method to enhance the assay throughput without compromising assay specificity.

2. Experimental

2.1. Chemical and reagents

2-(4-Chlorophenoxy)-2-methylpropionic acid ethyl ester (**I**) was obtained from The United States Pharmacopeial Convention (Rockville, MD, USA). 2-(4-Chlorophenoxy)-2-methylpropionic acid (**II**) and 4-chlorophenylacetic acid (**III**) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Certified grade ammonium formate, optima-grade methanol, acetonitrile and ethyl acetate, HPLC grade *n*-pentane and laboratory grade (90%) formic acid were obtained from Fisher (Fair Lawn, NJ, USA). *t*-Butyl methyl ether was purchased from E.M. Science (Gibbstown, NJ, USA). Human control plasma (heparinized and centrifuged) was ordered from Sera-Tec Biologicals (New Brunswick, NJ, USA). All chemicals were used as received. Water was purified by Milli-Q from Millipore (Bedford, MA, USA).

2.2. Apparatus

The HPLC system consisted of a Waters (Milford, MA, USA) 600E system controller and pump, 717 plus autosampler and Applied Biosystems (Foster City, CA, USA) 783A UV absorbance detector. The detector signal was acquired and processed using the Perkin Elmer Nelson Turbochrom Client/Server data acquisition system (Cupertino, CA, USA).

2.3. Chromatographic conditions

Separation of analytes was performed at ambient temperature using a BDS Hypersil C_{18} (5 μm, 150× 46 mm) analytical column proceeded by a matching BDS C_{18} (5 μm, 4×3.0 mm) guard column from Phenomenex (Torrance, CA, USA). Pump flow-rate was set at 1.5 ml/min. The autosampler was programmed with the carousel temperature set at 5 °C and injection volume at 20 μl. The absorbance wavelength on the UV detector was set at 230 nm. The isocratic HPLC method had a run time of 30 min using mobile phase 1, 29% acetonitrile–19% methanol–52% formic acid aqueous at pH 4.3, (v/v/v). The step-gradient HPLC method had a run time of 13 min using mobile phase 1 and 2, 56% acetonitrile–19% methanol–25% formic acid aqueous at pH 4.3, (v/v/v). The step-time was programmed as following: 0–5 min, mobile phase 1; 5–9 min, mobile phase 2; 9–13 min, mobile phase 1. The mobile phases were degassed by vacuum filtration using 0.2 μm Nylon-66 filters (Rainin, Woburn, MA, USA).

2.4. Preparation of calibration standards

The working standards of **I** and **II** at 10, 100 and 1000 μg/ml were prepared from their stock solution at 10 mg/ml in 50% acetonitrile–50% 50 m*M* ammonium formate (v/v). 10 μg/ml standard was used for obtaining UV absorbance spectrum. **III** was prepared at 500 μg/ml in the same solvent. All the standard solutions were prepared and used at room temperature and stored at 5 °C. Appropriate volumes

(12.5, 25.0 and 50.0 μl) of **I** and **II** working standards and control human plasma were mixed to obtain 500 μl of plasma calibration standards containing two analytes in the following concentrations: 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml. Aliquots of 40 μl of 500 μg/ml **III** and 500 μl of 1% formic acid were added to the plasma standards to adjust the pH to about 3.5 and mixed by vortex.

2.5. Preparation of quality control (QC) samples

QC stock solution of **I** was prepared from a separate weighing at 10 mg/ml in the above solvent. Appropriate volumes (12.5, 50 and 250 μl) of QC stock solution were transferred to 50 ml volumetric flasks and diluted to the mark with human control plasma to obtain plasma QCs at 2.5, 10.0 and 50.0 μg/ml concentrations. Plasma QCs of metabolite **II** were prepared similarly at the same concentrations. Plasma QC solutions were divided into 600 μl aliquots in 12×75 mm conical polypropylene tubes with caps (Sarstedt, Newton, NC, USA) and stored in the identical conditions with clinical samples at −70 °C.

The preparation of plasma calibration standard and QC samples were conducted in a plastic container with wet ice (~5 °C) to reduce the hydrolysis of **I** in human plasma.

2.6. Clinical sample collection

Blood samples about 10 ml were collected in the green-top heparinized NaF tubes, inverted three times and immediately centrifuged at 1449 *g* for 15 min at 4 °C. The plasma fractions were then transferred into the 4.5 ml nunc polypropylene tubes and placed in −70 °C. The total time from drawing blood to transferring the plasma to freezer should not exceed 30 min.

2.7. Clinical and QC sample preparation for analysis

Frozen plasma samples and QCs were thawed in room temperature water for about 5 min, mixed by vortex and then placed into a plastic container with wet ice (~5 °C). Aliquots of 500 μl were transferred to 12×75 mm conical polypropylene tubes which contained 500 μl of 1% formic acid and 40 μl of 500 μg/ml **III** and mixed. Clinical samples with calculated concentrations greater than the highest concentration of the standard calibration range were diluted with control plasma and then reanalyzed.

2.8. Solid-phase extraction (SPE) procedure

SPE was performed manually on the TomTec vacuum box (Model #196-503, Hamden, CT, USA) using 96-well SPEC·C_{18}AR extraction plate from Ansys Technologies (Lake Forest, CA, USA). The plate was conditioned with 250 μl of acetonitrile followed by 250 μl of Milli-Q water. Aliquots of 500 μl of acidified plasma standards, QCs or clinical samples were loaded onto the plate with slight house vacuum. After washed with 500 μl (×2) of 0.5% formic acid, the sample plate was centrifuged at 362 *g* for 5 min at 5 °C and then eluted with 250 μl of eluting solvent, 79% acetonitrile–20% water–1% formic acid, pH 2.5, (v/v/v). A volume of 20 μl of SPE eluent was injected directly onto the HPLC.

2.9. Recovery

Extraction recovery for **I** and **II** was calculated by the ratio of mean peak area ($n=3$) of plasma standard over reference standard at three concentrations, 2.5, 10.0 and 50.0 μg/ml. **III** was obtained similarly at working concentration ($n=9$). Plasma standards were prepared by the same procedures as calibration standards. Reference standards were prepared by spiking the appropriate volumes of working standards into the control plasma extract to achieve the same concentrations as plasma standards.

2.10. Stability

Stability of analytes in human plasma was examined by determining the drug remaining. Plasma standards of **I** or **II** at 5 and 50 μg/ml concentrations were prepared in 10 ml volume and kept at 5 °C or room temperature. Aliquots (500 μl) of standards were quantitated in replicate ($n=3$) every hour for 3 h to evaluate the stability of **I** at 5 °C and room temperature and stability of **II** at room temperature. The stability of **I** in acidified plasma at 5 °C was examined in the same way using 5 μg/ml of **I** in

control plasma containing 0.5% formic acid (pH 3.5).

Freeze–thaw stability of analytes was assessed by determining the accuracy of QCs to their nominal concentrations. Replicate sets of QCs at three concentrations were removed from the freezer and thawed at room temperature for about 5 min; triplicate sets at each concentration were quantitated and the others were stored at 5 °C for 30 min and frozen again for overnight. This process was repeated over three cycles.

The stability of analytes, which were extracted by SPE and kept in the HPLC autosampler, was determined by comparing the mean peak areas before and after 20 h storage in the autosampler at 5 °C. **I** and **II** were examined using plasma standards ($n=3$) at 5 and 50 μg/ml and **III** was by replicate ($n=6$) at working concentration.

2.11. Validation procedure

Assay intra-day accuracy and precision were accomplished by analyzing replicate five plasma standard curves ranging from 1.0 to 100.0 μg/ml for both analytes. The calibration curve was constructed using a weighted ($1/y$) linear regression of peak area ratios of analytes to I.S. versus nominal concentrations.

Duplicate sets of QCs at three concentrations were prepared with clinical samples and analyzed with a daily-prepared plasma standard curve. Prior to analysis with clinical samples, the intra-day accuracy and precision of QCs were determined by analyzing five replicates. The acceptance of clinical sample batch analysis was based on the accuracy of QC samples to their nominal concentrations (±20%).

3. Results and discussion

3.1. Sample preparation and SPE procedure

LLE was examined using several different conditions initially based on the literature [5,6]. 10% *n*-Pentane–90% ethyl acetate (PEA) gave about 70% and 94% recovery ($n=3$) for **I** and **II**, respectively, when 5 and 50 μg/ml plasma standards were handled under optimum conditions. The recovery using LLE was calculated as the peak area ratio of plasma standard over the reference standard, which contained the same amount of analytes in reconstitution solvent (mobile phase). Also, the neat standards were prepared in PEA at 5 and 50 μg/ml ($n=3$) and processed through the evaporation and reconstitution steps. The recovery of **I** and **II** in neat standard was about 75% and 97%, respectively. This result indicated that the low recovery of **I** was due to its volatility (BP: 148–150 °C), which caused analyte loss during the evaporation step. This loss could and did directly affect the assay's LLOQ, linearity and reproducibility of the calibration curve.

SPE allowed for rapid and specific separation without the evaporation step, thus it was investigated for isolation of **I**. The type of SPE material in 96-well plate, the pH of plasma sample and the eluting solvent were investigated for linearity, recovery and sensitivity. Ansys C_{18}, C_{18}·A (polar modified C_{18}) and C_{18}·AR (acid resistant C_{18}) SPE and Waters Oasis HLB (hydrophilic–lipophilic balanced copolymer) SPE were evaluated. The best recovery for **I** and **II** was obtained when loading a plasma sample at pH 3.5 onto the Ansys C_{18}·AR plate, washing with 0.5% formic acid, and eluting with 79% acetonitrile–20% water–1% formic acid (v/v/v). Assay recoveries for **I** and **II** at 2.5, 10.0 and 50.0 μg/ml vary from 94.5 to 100.9% and 104.3 to 104.6%, respectively, with precision less than 3% coefficient of variation (C.V.) for triplicate analysis. **III** has 100.4% recovery at working concentration. This procedure was validated for assay intra-day accuracy and precision and assay specificity over the calibration range of 1.0 to 100.0 μg/ml.

During the investigation of extraction process, it was observed that **I** quickly hydrolyzed to **II** in human plasma at room temperature. Therefore, sample preparation was conducted at 5 °C (in a plastic container half-full with wet ice) to minimize the reaction.

3.2. Chromatography

Using reversed-phase chromatography, the endogenous materials of control plasma eluted in the retention window of **II** and not **I**. To achieve the assay specificity, different analytical columns and various mobile phases were evaluated at a flow-rate

of 1.0 or 1.5 ml/min and UV absorbance wavelength at 223 nm. Chromatographic specificity was achieved using an Aquasil C_{18}, 5 μm, 100×4.6 mm column from Keystone Scientific (Bellefonte, PA, USA) with a mobile phase of 29% acetonitrile–19% methanol–52% formic acid aqueous at pH 3.4 (v/v/v) with a set flow-rate of 1.5 ml/min. However, under these chromatographic conditions, the precision of an intra-day validation of **I** was not successful (precision >10% C.V.). Stability studies of **I** eliminated the possibility of degradation during sample preparation and sample analysis. It was observed that the retention time of **I** shifted when using different lots of the analytical columns. Thus, the Aquasil C_{18} column was considered not suitable for **I** analysis. Assay specificity and consistent chromatographic retention were achieved with BDS Hypersil C_{18}, 5 μm, 150×4.6 mm column from Phenomenex and 29% acetonitrile–19% methanol–52% formic acid aqueous at pH 4.3 (v/v/v) as mobile phase. In addition, UV absorbance wavelength was changed from 223 to 230 nm to enhance specificity. The maximum wavelength for **I** is 226 nm. **II** gives a very similar spectrum. The chromatograms of **I** and **II** with I.S. in human plasma using isocratic HPLC method are shown in Fig. 2a.

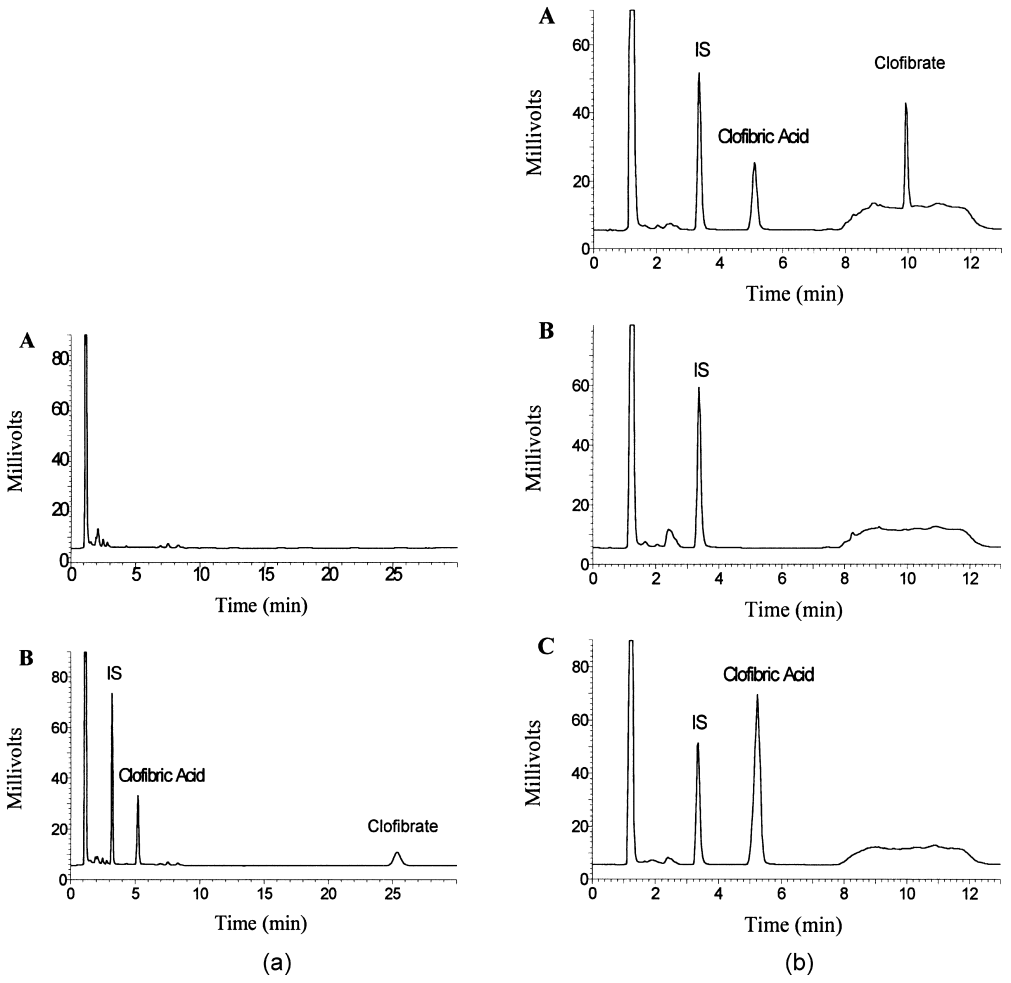

Fig. 2. (a) Chromatogram of (A) a control human plasma blank and (B) 10 μg/ml plasma standard of **I** and **II** with I.S. (**III**) using isocratic HPLC method. (b) Chromatogram of (A) 10 μg/ml plasma standard of **I** and **II** with I.S. (**III**), (B) a predose plasma sample and (C) a postdose plasma sample using the step-gradient HPLC method.

Because the isocratic HPLC method had a 30 min run time, a step-gradient method was investigated to shorten the elution time of **I**. The isocratic mobile phase was used as mobile phase 1 to maintain the assay specificity. After metabolite **II** eluted from analytical column, the mobile phase was switched to a higher organic content mobile phase 2, 56% acetonitrile–19% methanol–25% formic acid aqueous at pH 4.3 (v/v/v) to elute **I**. Then, the mobile phase was changed back to mobile phase 1 to equilibrate the column and wait for the next injection. The run time was decreased to 13 min with the following gradient step-times: 0–5 min, mobile phase 1; 5–9 min, mobile phase 2; 9–13 min, mobile phase 1 (Fig. 2b).

The assay was specific for **I**, **II** and I.S. in human plasma under both isocratic and gradient conditions. Eight lots of commercial control human plasma and the predose subjects from the clinical study confirmed that no endogenous material eluted in the retention windows of both analytes and I.S.

3.3. Linearity, accuracy and precision

Intra-day accuracy and precision of five sets of calibration standard curves prepared from five different lots of control plasma using the isocratic HPLC method are listed in Table 1A. The step-gradient method was validated similarly (Table 1B). Assay accuracy did not deviate by more than 15% of the nominal value at each concentration and precision did not exceed 10% C.V. except for LLOQ where it did not exceed 15%. Intra-day validation data of QCs at three concentrations are also indicated in Table 1A. The mean QC value did not deviate by more than 15% of the nominal and precision did not exceed 10% C.V.

Inter-day accuracy and precision were calculated

Table 1A
Intra-day accuracy, precision and linearity of the isocratic HPLC method for **I** and **II** in human plasma

Standard	Nominal conc. (μg/ml)	Mean ($n=5$) calc. conc. (μg/ml)	Accuracy[a] (%)	Precision[b] (% C.V.)	Linearity
I	1	1.14	114.2	13.31	$r^2=0.9970$
	2.5	2.48	99.4	7.12	slope = 0.04025
	5	4.66	93.2	6.81	intercept = −0.00985
	10	9.45	94.5	7.67	
	25	25.20	100.8	3.54	
	50	49.91	99.8	1.73	
	100	101.15	101.2	6.04	
II	1	0.92	92.4	6.96	$r^2=0.9986$
	2.5	2.60	103.9	1.94	slope = 0.05251
	5	5.16	103.2	3.29	intercept = 0.00275
	10	9.98	99.8	2.43	
	25	25.61	102.4	3.11	
	50	50.19	100.4	2.02	
	100	99.27	99.3	4.20	
QC					
I	2.5	2.13	85.1	6.57	
	10	8.87	88.7	4.57	
	50	44.48	89.0	2.43	
II	2.5	2.46	98.4	4.96	
	10	9.51	95.1	1.40	
	50	46.95	93.9	0.87	

[a] Percent of mean calculated concentration over nominal concentration.
[b] Coefficient of variation of peak area ratio (analytes/I.S.).

Table 1B
Intra-day accuracy, precision and linearity of the step-gradient HPLC method for **I** and **II** in human plasma

Standard	Nominal conc. (μg/ml)	Mean ($n=5$) calc. conc. (μg/ml)	Accuracy[a] (%)	Precision[b] (% C.V.)	Linearity
I	1	1.03	102.6	2.40	$r^2=0.9995$
	2.5	2.49	99.8	1.37	slope = 0.02350
	5	4.95	99.0	3.58	intercept = −0.00305
	10	9.76	97.6	1.94	
	25	25.54	102.2	3.17	
	50	49.88	99.8	2.65	
	100	99.94	99.9	0.28	
II	1	1.02	101.8	2.74	$r^2=0.9996$
	2.5	2.55	101.9	4.45	slope = 0.02899
	5	4.85	97.0	1.55	intercept = −0.00363
	10	9.88	98.8	0.77	
	25	25.75	103.0	1.42	
	50	49.14	98.3	1.24	
	100	100.41	100.4	0.41	

[a] Percent of mean calculated concentration over nominal concentration.
[b] Coefficient of variation of peak area ratio (analytes/I.S.).

Table 2
Inter-day accuracy and precision of QC samples using the step-gradient HPLC method

QC	Nominal conc. (μg/ml)	Mean[a] ($n=6$) calc. conc. (μg/ml)	Accuracy[b] (%)	Precision[c] (% C.V.)
I	2.5 (LQC)	2.11	84.5	6.06
	10 (MQC)	9.14	91.4	8.14
	50 (HQC)	47.08	94.2	14.62
II	2.5 (LQC)	2.57	102.6	3.62
	10 (MQC)	10.14	101.5	4.19
	50 (HQC)	48.74	97.5	4.37

[a] From six measurements conducted at 3 different days over 5 months.
[b] Percent of mean concentration over nominal concentration.
[c] Coefficient of variation of peak area ratio (analytes/I.S.).

Table 3
Freeze–thaw stability of **I** and **II** in human plasma

QC	Cycle	Mean calculated concentration[a] (μg/ml)		
		2.5 (LQC)	10 (MQC)	50 (HQC)
I	1	2.13	8.87	44.48
	2	2.42	9.23	43.39
	3	2.21	8.03	40.46
	Mean	2.25	8.71	42.78
	Accuracy (%)	90.10	87.10	85.60
	Precision (% C.V.)	6.65	7.07	4.86
II	1	2.46	9.51	46.95
	2	2.28	8.88	44.78
	3	2.70	10.10	50.02
	Mean	2.48	9.50	47.25
	Accuracy (%)	99.20	95.00	94.50
	Precision (% C.V.)	8.50	6.42	5.57

[a] 1st freeze–thaw cycle was $n=5$ and 2nd and 3rd cycles were $n=3$.

from the average measurements of QCs at three concentrations over 5 months (Table 2). The clinical sample storage stability was demonstrated from the inter-day QC concentrations. The accuracy of QC data (within 20% of the nominal value) indicated that both analytes were stable in plasma for at least 5 months when stored at −70 °C.

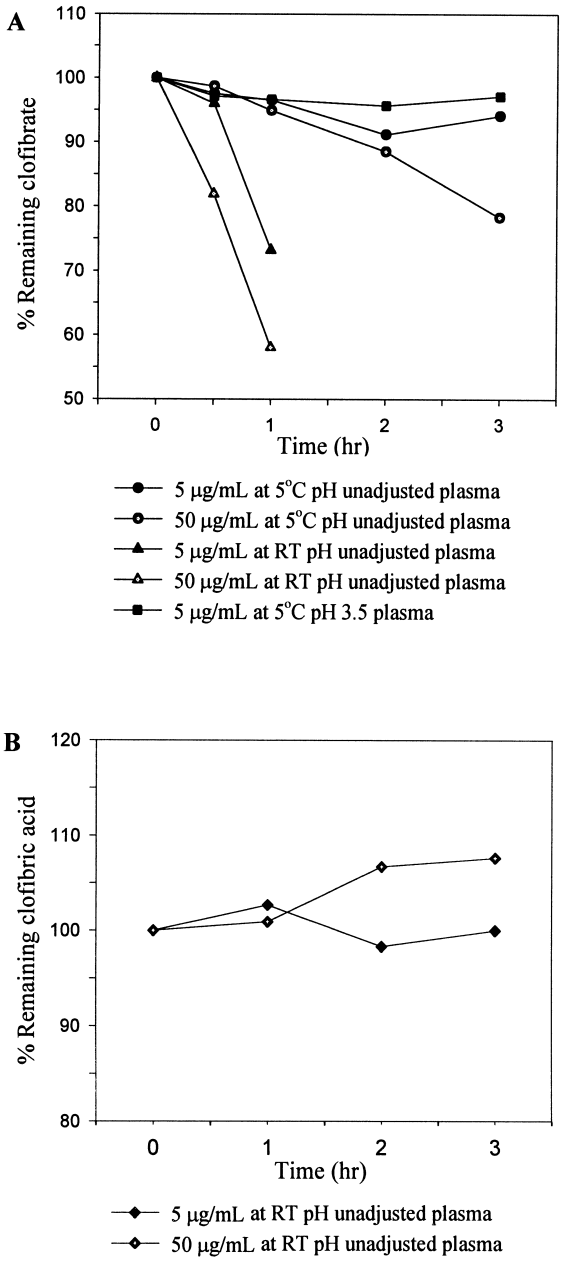

Fig. 3. Stability of **I** (A) and **II** (B) in human plasma. (Each point is the mean of triplicate analysis.).

3.4. Stability

I quickly hydrolyze to **II** in human plasma at room temperature. The stability of **I** was studied under two conditions: pH unadjusted plasma at 5 °C versus room temperature and pH 3.5 plasma versus pH unadjusted plasma at 5 °C (Fig. 3). This study indicates that low temperature significantly improves the stability of **I** in plasma and the stability of **I** in acidified human plasma is better than in pH unadjusted plasma at 5 °C. The stability of **II** was evaluated at 5 and 50 μg/ml and the results indicate **II** is stable in human plasma at room temperature for at least 3 h.

Freeze–thaw stability data of QCs are listed in Table 3 and demonstrate that both analytes are stable in human plasma over three cycles. There is no observable degradation of **I** after each freeze–thaw cycle and the differences in mean concentrations between each cycle appear to be due to inter-day assay variability. Stability of SPE extracted analytes during sample analysis is given in Table 4 and shows that **I**, **II** and **III** are able to be stored in the autosampler programmed at 5 °C for 20 h with less than 3% changes.

Stability of the clofibric acid glucuronide, the metabolite of **II**, was not evaluated since its reported levels in healthy subjects were below limits of detection [7–9].

3.5. Application

Healthy young males were administered 1 g of **I** orally twice daily after meals at approximately 8:00 a.m. and 8:00 p.m. from day 1 to day 14. Plasma samples were collected prior to dosing at approximately 8:00 a.m. on days 1, 13, 14 and 15. They were analyzed by the step-gradient method and the results are displayed in Fig. 4. Plasma concentrations of **I** are lower than LLOQ in all postdose samples and the plasma level of **II** is 30 to 120 μg/ml. These results suggested that **I** was completely converted to **II** in the human body at the designated plasma sample collection times.

Table 4
Stability of SPE analytes at 5 °C in the autosampler

Standard	Nominal conc. (μg/ml)	Initial		20 h		Change[b] (%)
		Mean peak area[a]	% C.V.	Mean peak area[a]	% C.V.	
I	5	9.86E+04	3.42	9.86E+04	3.99	0.05
	50	9.69E+05	7.68	9.92E+05	7.63	2.30
II	5	1.36E+05	5.92	1.36E+05	5.40	0.41
	50	1.38E+06	3.09	1.42E+06	2.90	2.56
III	40	5.23E+05	8.99	5.33E+05	10.60	1.86

[a] **I** and **II** were $n=3$ and **III** was $n=6$.
[b] [(20 h mean peak area − initial mean peak area)/initial mean peak area × 100(%)].

4. Conclusion

Precise and accurate HPLC assays have been developed for simultaneous determination of **I** and its active metabolite **II** in human plasma using a 96-well SPE plate. SPE effectively enhanced the linearity of the calibration standard curve and assay recovery; while using LLE to isolate a liquid analyte with a low boiling point like **I** is not recommended due to large losses of analyte during the evaporation step. The stability of **I** in human plasma was improved by decreasing the sample preparation temperature (5 °C) and adjusting the plasma pH to 3.5. The assay has been shown to be specific using many different lots of commercial control plasma and predose subject samples and has been applied to support a clinical study.

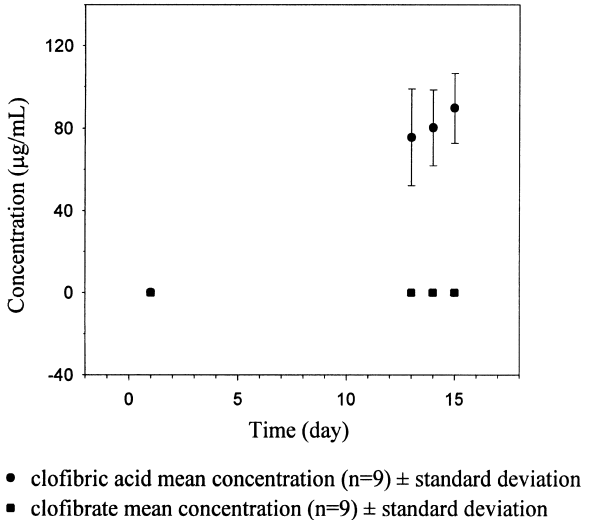

Fig. 4. Plasma concentrations (mean ± S.D.) of **I** and **II** from nine healthy young males.

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