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Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 101–108

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Simultaneous determination of pioglitazone and its two active metabolites in human plasma by LC–MS/MS

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Received 30 July 2002; accepted 26 March 2003

Abstract

A liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous determination of pioglitazone (PIO) and its two metabolites: M-III (keto-derivative) and M-IV (hydroxy-derivative) in human plasma. Human plasma samples of 0.2 ml were extracted by a single step liquid–liquid extraction procedure and analyzed using a high performance liquid chromatography (HPLC) electrospray tandem mass spectrometer system. The compounds were eluted isocratically on a C-18 column, ionized using a positive ion atmospheric pressure electrospray ionization source and analyzed using multiple reaction monitoring mode. The ion transitions monitored were m/z 357 → 134 for PIO, m/z 371 → 148 for M-III, m/z 373 → 150 for M-IV and m/z 413 → 178 for the internal standard. The chromatographic run time was 2.5 min per injection, with retention times of 1.45, 1.02 and 0.95 min for PIO, M-III and M-IV, respectively. The calibration curves of pioglitazone, M-III and M-IV were well fit over the range of 0.5–2000 ng/ml ($r^2 > 0.998759$) by using a weighted ($1/x^2$) quadratic regression. The inter-day precisions of the quality control samples (QCs) were ≤ 10.5% (N = 15), coefficient of variation (CV) and the inter-day accuracy (%Nominal) ranged from 84.6 to 103.5% for PIO, 94.4 to 104.0% for M-III, and 96.8 to 101.0% for M-IV. All three analytes demonstrated acceptable short-term, long-term, and freeze/thaw stability. The method is simple, rapid and rugged, and has been applied successfully to sample analysis for clinical studies.
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Keywords: Pioglitazone; Keto-derivative (M-III); Hydroxy-derivative (M-IV); LC–MS/MS

1. Introduction

Pioglitazone hydrochloride, (±)-5-{4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl}-2,4-thiazolidinedione hydrochloride salt, is an oral antidiabetic agent that has been shown to affect abnormal glucose and lipid metabolism associated with insulin

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doi:10.1016/S0731-7085(03)00344-3

resistance by enhancing insulin action on peripheral tissues in animal models [1–5]. Pioglitazone (PIO) is extensively metabolized by hydroxylation and oxidation. Metabolites M-III (keto derivative of pioglitazone) and M-IV (hydroxy derivative of pioglitazone) are pharmacologically active in animal models of type 2 diabetes [6]. At steady-state, M-III and M-IV reach serum concentrations equal to or greater than pioglitazone [6]. Analysis of PIO and its metabolites in biological fluids by high performance liquid chromatography (HPLC) with ultraviolet detection has appeared in the literature [7–9]. However, previously published HPLC assays lack specificity, sufficient sensitivity (LLOQ: 10–50 ng/ml using a sample volume of 0.2–0.5 ml) and require long analytical run times (over 20 min), which make the LC-UV method impractical for routine analysis of large numbers of clinical samples. Quantitative LC–MS/MS methods suitable for the routine analysis of PIO and its metabolites have not been reported. Therefore, this paper describes a sensitive, specific, and rapid LC–MS/MS method for the simultaneous determination of PIO and its two active metabolites (M-III and M-IV) in human plasma.

2. Experimental

2.1. Chemicals and reagents

PIO (> 99% purity), M-III (98.6% purity), M-IV (99.5% purity) and internal standard (5-(4-(2-(5-(2-methyl-1,3-dioxolan-2-yl)-2-pyridyl)ethoxy)-benzylidene)-2,4-thiazolidinedion, IS; 99.0% purity) were synthesized at Avantix Laboratories, Inc. (New Castle, DE, USA). The chemical structures of PIO, M-III, M-IV and IS, a structure analog of PIO, are shown in Fig. 1. Type I water was from UHQ-PS (High Wycombe, Bucks, UK). Methyl *t*-butyl ether, *n*-butyl chloride, acetonitrile, methanol, and ammonium hydroxide were from Fisher Scientific (St. Louis, MO, USA). Ammonium acetate and dimethylformamide were from Aldrich (Milwaukee, WI, USA), and triflouroacetic acid (TFA) was from Burdick & Jackson (Muskegon, MI, USA). Blank human sodium heparin plasma was from Bioreclamation Inc. (Hicksville, NY, USA) and was stored in a freezer at −20 °C. All mobile phase solvents were HPLC grade and all other reagents were analytical reagent grade.

2.2. Calibration standards and quality control samples

Standards and quality control samples (QCs) were made from two separate stock solutions (1 mg/ml, PIO in methanol/dimethylformamide, 70:30 v/v; M-III and M-IV in methanol). Working calibration standards at concentrations of 0.5, 1, 5, 20, 200, 1000 and 2000 ng/ml in plasma were prepared fresh daily. Five levels of QC samples, 0.5, 1, 20, 1000 and 2000 ng/ml, were prepared in plasma for the determination of inter-day accuracy and precision, aliquoted, and stored frozen at −20 °C. QCs exceeding the upper limit of quantitation (ULOQ) were prepared at 5000 ng/ml for the determination of dilution integrity. Stock solution of internal standard (1 mg/ml) was prepared in methanol/dimethylformamide, 80:20 (v/v) and 50 ng/ml of IS working solution was prepared in acetonitrile.

2.3. LC–MS/MS methods

LC–MS/MS analyses were performed using a Hewlett Packard 1100 HPLC system (Wilmington, DE, USA) coupled to a Micromass Quattro LC triple-quadrupole mass spectrometer (Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI^+) with multi reaction monitoring (MRM). The analytical column was a MetaChem Polaris C18-A, 50 × 2 mm, 3 μm (Lake Forest, CA, USA). The mobile phase consisted of acetonitrile:water (60:40) with 10 mM ammonium acetate and 0.02% TFA at an isocratic flow rate of 0.2 ml/min. The sample injection volume was 10 μl and run time was 2.5 min.

Sensitivity of MRM was optimized by infusing a mixture of 50 ng/ml PIO, M-III and M-IV in the mobile phase. The capillary voltage was maintained at 3.5 kV. The cone and the extractor voltages were set to 35 and 3 V, respectively. The desolvation and ion source temperatures were 400 and 80 °C, respectively. Ions were activated and

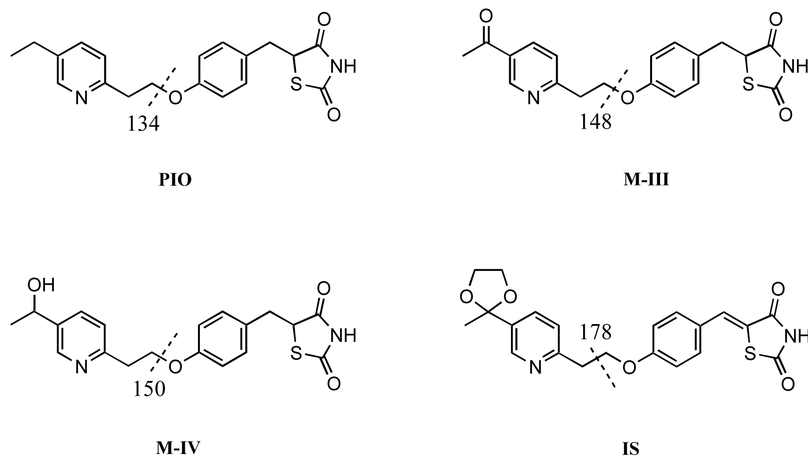

Fig. 1. Chemical structures of PIO, M-III, M-IV and the internal standard (IS).

fragmented at a collision energy of 35 eV and at an indicated argon pressure of 1.9×10^{-3} Torr. To assay all analytes, both quadrupoles were maintained at unit resolution and the transitions (precursor to daughter) monitored were *m/z* 357→134 for PIO, *m/z* 371→148 for M-III, *m/z* 373→150 for M-IV and *m/z* 413→178 for IS. The fragmentation patterns are shown in Fig. 1. The dwell time for each transition was 200 ms and the interchannel delay was 20 ms.

MRM data were acquired and the chromatograms were integrated using MassLynx™ NT, version 3.2 software. A weighted 1/concentration^2 quadratic regression was used to generate calibration curves from standards and calibration curves were then used to calculate the concentrations of samples.

2.4. Sample preparation

All samples, QCs, and standards with a sample volume of 0.2 ml spiked with 100 μl of IS (50 ng/ml in acetonitrile) were made acidic by addition of 1 ml of aqueous 0.1 M ammonium acetate adjusted the pH to 4 with acetic acid, and were extracted into 4 ml of 1:1 (v/v) methyl *t*-butyl ether: *n*-butyl chloride. The extraction tubes were shaken at high speed for 20 min followed by centrifugation at 4000 rpm for 20 min. The organic phase was transferred to clean glass tubes and evaporated to dryness in a 45 °C water bath under a nitrogen stream. The residues were dissolved in 100 μl of mobile phase and vortexed for 1 min. After transfer into glass inserts of autosampler vials and centrifugation for 5 min at 4000 rpm, an aliquot of 10 μl of each sample was injected into the LC–MS/MS system.

2.5. Validation of the LC–MS/MS method

The method was validated for accuracy, precision, sensitivity, specificity, calibration curve range, and reproducibility according to the FDA guideline for bioanalytical methods validation [10] over a concentration range of 0.5–2000 ng/ml using seven calibration standards, each containing the three analytes of interest, and five replicates of QC samples at each concentration level in three separate runs. Each run might also contain additional samples such as stability samples for processing and storage.

Analyte stability was tested using QC samples for multiple freeze/thaw (F/T) cycles, on the bench at room temperature (short-term stability), or at −20 °C in the freezer (long-term storage). Post-preparative stability and stock solution stability were also determined. The extraction recoveries of PIO, M-III and M-IV were calculated by comparing the peak areas of extracted plasma standards to the peak areas of post-extraction plasma blanks spiked at corresponding concentrations. The overall absolute recovery from human plasma was

determined by comparing the peak areas of extracted plasma standards to those prepared in mobile phase. The method specificity was evaluated by screening six lots of blank sodium heparin plasma.

3. Results and discussion

3.1. LC–MS/MS method

The MetaChem Polaris C18-A column used for separation provided good retention for polar compounds and maintained good peak shapes. In order to minimize quantitation bias caused by matrix effect, an IS with identical or similar retention time as the analyte but resolved by tandem mass spectrometry is usually preferred. Therefore, a structural analog of pioglitazone was synthesized as the IS. It was demonstrated that the IS was stable during sample preparation, under LC–MS/MS conditions, and had similar retention time as PIO, M-III and M-IV.

3.2. Specificity, sensitivity and calibration curve range

Human blank plasma samples from six different subjects were extracted and analyzed for PIO, M-III and M-IV as a true blank (double blank), or spiked with IS, or with PIO and metabolites as a single blank. There were no endogenous peaks that interfered with the quantitation of PIO, M-III, M-IV or IS. There was no interference from IS contributing to the PIO, M-III or M-IV *m/z* channels or from PIO, M-III or M-IV contributing to the IS *m/z* channel. The ratio of signal to noise obtained from an extracted lower limit of quantitaion (LLOQ) sample (0.5 ng/ml) was at least 30 for PIO, M-III and M-IV. There was no significant lot-to-lot variation in matrix effect and no carry-over from ULOQ to blank sample observed. Calibration curves were well fit in the concentration range of 0.5–2000 ng/ml using a quadratic regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) for PIO, M-III and M-IV. Representative chromatograms of blank human plasma spiked with PIO, M-III and M-IV at LLOQ and IS, blank human plasma, blank human plasma spiked with IS only, and blank human plasma spiked with PIO, M-III and M-IV only are shown in Figs. 2–5.

3.3. Precision, accuracy and dilution integrity

Table 1 shows the validation data on accuracy and precision of each standard concentration. The coefficients of variation (CV, N = 6) of the back-calculated calibration standards at 0.5 ng/ml were 4.4, 6.3 and 3.0% for PIO, M-III and M-IV, respectively, and at 2000 ng/ml were 5.9, 5.4 and 4.8%, respectively. The precision and accuracy data for QCs are summarized in Table 2. For

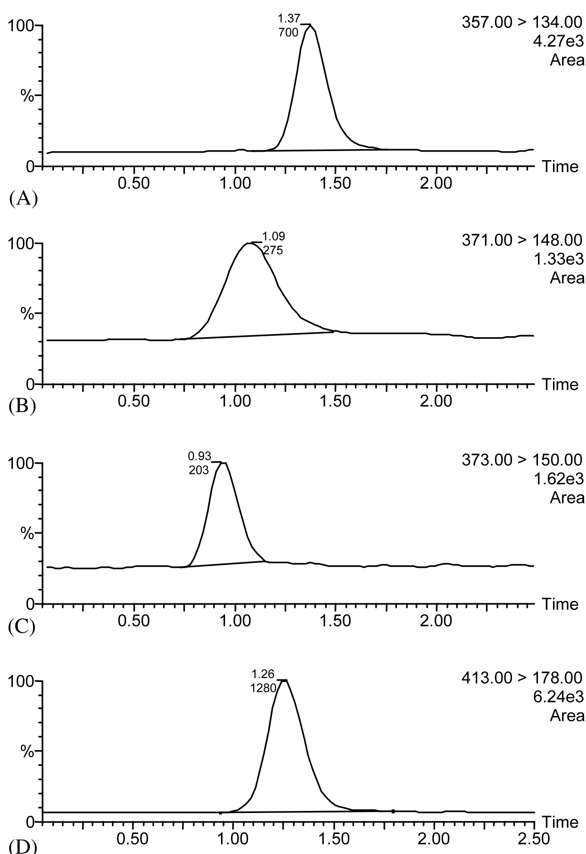

Fig. 2. Chromatograms of an extracted human plasma spiked with PIO, M-III and M-IV at LLOQ (0.5 ng/ml). (A) PIO channel: *m/z* 357 → 134; (B) M-III channel: *m/z* 371 → 148; (C) M-IV channel: *m/z* 373 → 150; (D) IS channel: *m/z* 413 → 178.

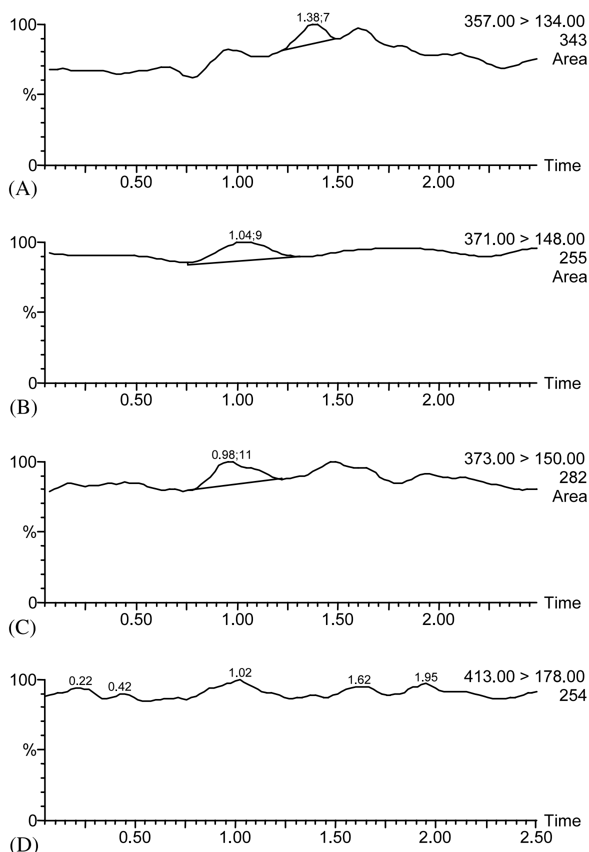

Fig. 3. Chromatograms of an extracted blank human plasma sample. (A) PIO channel: m/z 357→134; (B) M-III channel: m/z 371→148; (C) M-IV channel: m/z 373→150; (D) IS channel: m/z 413→178.

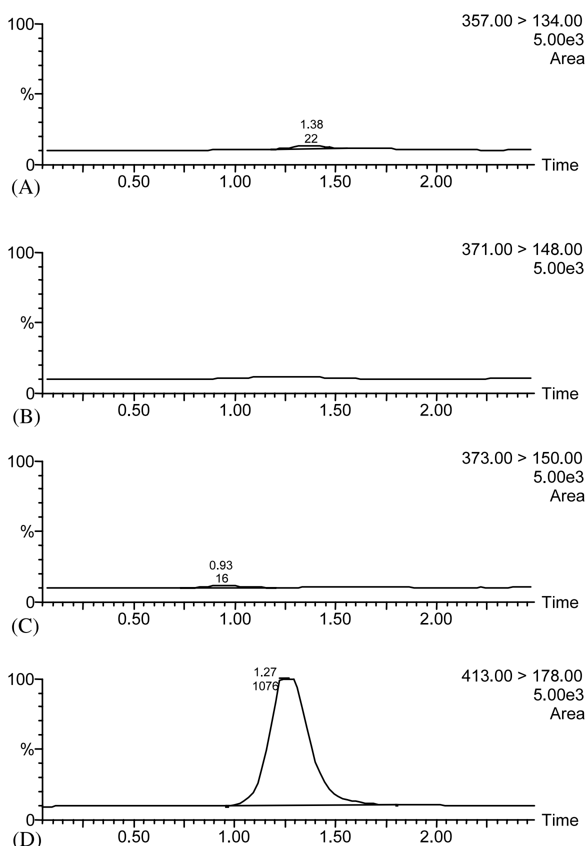

Fig. 4. Chromatograms of an extracted human plasma spiked with internal standard only. (A) PIO channel: m/z 357→134; (B) M-III channel: m/z 371→148; (C) M-IV channel: m/z 373→150; (D) IS channel: m/z 413→178.

QCs at 0.5 (LLOQ) and 2000 ng/ml, inter-assay CV values were 4.3 and 7.5%, respectively, for PIO, 4.6 and 10.5%, respectively, for M-III, and 3.0 and 7.5%, respectively, for M-IV. The %Nominal ranged from 84.6 (at LLOQ) to 103.5% for PIO, 94.4 to 104.0% for M-III, and 96.8 to 101.0% for M-IV. Intra-assay CV, for QCs at 0.5 and 2000 ng/ml, was 1.8 and 1.2%, respectively, for PIO, 3.8 and 1.0%, respectively, for M-III and 1.2 and 1.0%, respectively, for M-IV. The %Nominal ranged from 88.4 to 104.1% for PIO, 97.6 to 102.0% for M-III and 95.9 to 101.2% for M-IV. The "tight" CV and %Nominal values indicated reproducible LC–MS/MS conditions and that the assay is consistent and reliable. For partial volume analysis, QC samples (5000 ng/ml) were diluted 10-fold with blank plasma prior to extraction. The dilution integrity data showed the CV values were 10.6, 9.2 and 10.3% for PIO, M-III and M-IV, respectively, with a %Nominal of 97.0, 103.2, and 98.9% for PIO, M-III and M-IV, respectively. These results support sample dilution up to 10-fold for analysis.

3.4. Recovery and matrix effect

The extraction recovery was determined by comparing the peak areas of extracted plasma standards at 0.5, 20 and 2000 ng/ml to the peak areas of post-extraction plasma blanks spiked at

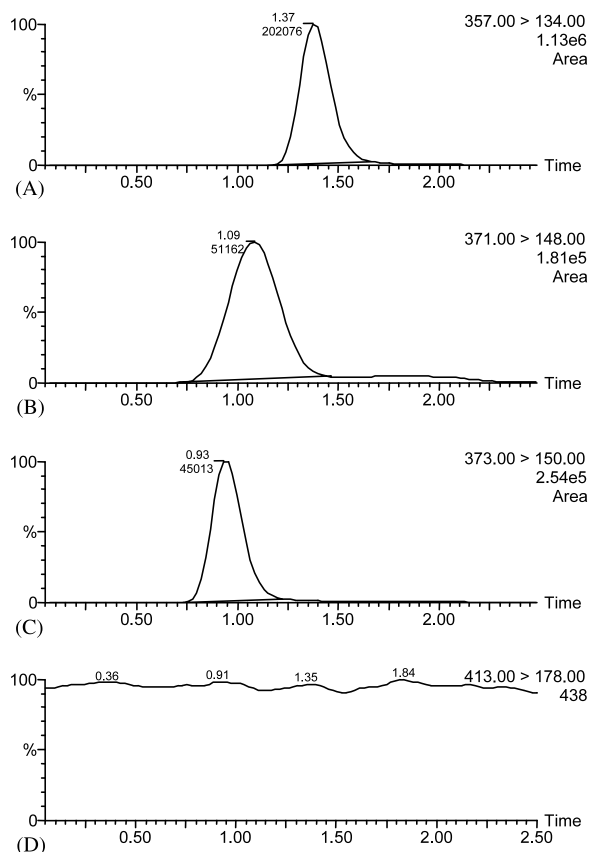

Fig. 5. Chromatograms of an extracted human plasma spiked with only PIO, M-III and M-IV. (A) PIO channel: m/z 357 → 134; (B) M-III channel: m/z 371 → 148; (C) M-IV channel: m/z 373 → 150; (D) IS channel: m/z 413 → 178.

corresponding concentrations. Extraction recovery from human plasma ranged from 88.2 to 94.8% for PIO, 95.2 to 96.2% for M-III, 85.8 to 91.2% for M-IV, and 90.4 to 95.1% for IS, respectively. The overall absolute recovery ranged from 62.5 to 71.1% for PIO, 28.3 to 29.0% for M-III, 47.9 to 55.4% for M-IV and 42.0 to 45.7% for IS, respectively. The lower overall absolute recoveries from extracted plasma were due to matrix effects. However, matrix effects did not cause quantitation bias as evidenced by the "tight" CV and %Nominal values obtained with the LLOQ QCs. Therefore, attempts to further improve the matrix effect were not pursued.

3.5. Stability of the analytes

The stability tests of the analytes were designed to cover anticipated conditions that clinical samples may experience. Stability data are summarized in Table 3. Briefly, three freeze/thaw cycles and ambient temperature storage of the QC samples up to 4 h prior to sample preparation appeared to have no effect on the quantitation of PIO, M-III and M-IV. QCs stored in a freezer at −20 °C remained stable for at least 5 months. Extracted analytes were allowed to stand at ambient temperature in mobile phase for 24 h prior to LC–MS/MS analysis, with no observed effect on quantitation. Stability of stock solutions was also investigated. When stock solutions of PIO in a mixture of methanol–dimethylformamide (70:30) or M-III and M-IV in methanol were stored at a nominal temperature of 4 °C for 1 month or at room temperature for 6 h, the analytes were stable.

Table 1
Precision and accuracy of calibration standards (N = 6)

Nominal (ng/ml)	PIO			M-III			M-IV		
	Mean	%CV	%Nominal	Mean	%CV	%Nominal	Mean	%CV	%Nominal
0.5	0.563	4.4	112.6	0.475	6.3	95.0	0.504	3.0	100.8
1	0.968	4.0	96.8	1.02	2.9	102.0	0.984	2.7	98.4
5	5.05	5.0	101.0	5.05	3.4	101.0	4.88	3.3	97.6
20	20.0	4.1	99.8	19.8	2.5	99.0	19.4	1.7	97.1
200	207	4.2	103.7	210	1.7	105.2	209	1.1	104.4
1000	965	2.6	96.5	966	4.5	96.6	983	2.5	98.3
2000	2047	5.9	102.3	1977	5.4	98.9	2013	4.8	101.4

Table 2
Precision and accuracy of QCs

Nominal (ng/ml)	PIO			M-III			M-IV		
	Mean	%CV	%Nominal	Mean	%CV	%Nominal	Mean	%CV	%Nominal
Intra-assay ($N = 5$)									
0.5	0.442	1.8	88.4	0.498	3.8	99.6	0.506	1.2	101.2
1	0.935	2.1	93.5	1.02	2.0	102.0	0.959	2.5	95.9
20	19.9	1.7	99.3	20.4	2.3	101.9	19.2	1.3	96.1
1000	956	1.3	95.6	976	1.9	97.6	963	1.2	96.3
2000	2082	1.2	104.1	1973	1.0	98.7	1964	1.0	98.2
Inter-assay ($N = 15$)									
0.5	0.423	4.3	84.6	0.503	4.6	100.6	0.499	3.0	99.8
1	0.977	4.8	97.7	1.04	4.8	104.0	1.01	5.0	101.0
20	20.5	4.0	102.5	20.7	2.8	103.3	20.1	4.0	100.5
1000	965	2.7	96.5	944	4.9	94.4	968	2.3	96.8
2000	2070	7.5	103.5	2005	10.5	100.2	1989	7.5	99.4

4. Conclusions

An LC–MS/MS method for quantitation of pioglitazone and its two active metabolites in human plasma has been successfully developed and validated. The method is simple, rapid, and rugged. The lower limit of quantitation was 0.5 ng/ml for PIO, M-III and M-IV using a 0.2 ml sample aliquot. The method is suitable for routine quantitation of PIO, M-III and M-IV in human plasma

Table 3
Stability of pioglitazone, M-III and M-IV

	Concentration (ng/ml)								
	PIO			M-III			M-IV		
	0.5	20	2000	0.5	20	2000	0.5	20	2000
4 h short-term at 25 °C									
Mean ($n = 3$)	0.501	19.5	2064	0.526	19.5	1961	0.506	20.3	1966
%CV	7.0	2.5	1.8	7.8	4.9	2.8	0.6	2.9	2.8
%Nominal	100.2	97.4	103.2	105.2	97.6	98.1	101.2	101.7	98.3
5 months at −20 °C									
Mean ($n = 3$)	0.512	18.5	1690	0.453	20.3	2006	0.567	21.8	2095
%CV	6.8	2.8	0.8	NA	2.1	1.8	7.6	1.1	3.5
%Nominal	102.4	92.4	84.5	90.6	101.6	100.3	113.4	109.1	104.8
Three freeze/thaw cycles									
Mean ($n = 3$)	0.523	19.8	2140	0.520	19.7	2077	0.518	20.7	2040
%CV	10.1	4.0	3.0	4.0	3.1	3.7	NA	3.3	2.1
%Nominal	104.6	98.9	107.1	104.0	98.6	103.9	103.6	103.6	102.0
24 h post-preparative									
Mean ($n = 3$)	0.504	19.1	2033	0.524	19.3	1938	0.492	19.6	2003
%CV	3.2	1.3	1.3	2.4	2.2	1.7	3.0	3.0	2.0
%Nominal	100.8	95.4	101.7	104.8	96.4	96.9	98.4	97.9	100.2

NA, not applicable due to one sample lost during extraction.

over a concentration range of 0.5–2000 ng/ml, and has been successfully used to analyze samples from clinical trials of drug–drug interaction studies.

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