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Simultaneous quantitation of pioglitazone and its metabolites in human serum by liquid chromatography and solid phase extraction

W.Z. Zhong*, M.G. Williams

Drug Metabolism Research, Pharmacia & Upjohn, Inc. Kalamazoo, MI 49001, USA

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Abstract

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of pioglitazone (U-72107) and its potential metabolites (M-1 to M-6) in human serum was developed. The method involved a solid phase extraction (SPE) of pioglitazone, its metabolites, and the internal standard (U-92573) from serum using C_{18} SPE columns with an elution solvent of 0.5 ml of acetonitrile–water (35:65, v/v). Separation of the eight analytes was achieved within 20 min using a reversed-phase Zorbax RX-C_8 analytical column (250 mm × 4.6 mm i.d., 5 μm particle size) with a mobile phase of acetonitrile–water (40:60, v/v) containing 3 ml acetic acid per liter mobile phase (apparent pH 5.5). An ultraviolet detector operated at 269 nm was used with a linear response observed from 0.02 to 2 μg ml^{-1} for these analytes except for M-4 which was best fitted with a polynomial regression. Limit of quantitation was found to be 0.02 μg ml^{-1} for pioglitazone, M-3, M-5, and M-6; 0.04 μg ml^{-1} for M-2 and M-4; and 0.5 μg ml^{-1} for M-1 when using a 0.5 ml serum sample for extraction. Obtained from the method validation, intra- and inter-assay precision was $\leqslant$9% and accuracy ranged from −8.2 to 13.4% for all analytes. The applicability of this method has been demonstrated by successfully analyzing clinical serum samples. The strategies in the HPLC characterization and in the SPE procedure development for this method are discussed as well.

Keywords: HPLC; Solid phase extraction; Human serum; Metabolites

1. Introduction

Pioglitazone hydrochloride, 5-{4-[2-(5-ethyl-2-pyridyl)ethoxy] benzyl}-2,4-thiazolidinedione hydrochloride salt (U-72107A, AD-4833) (Fig. 1), was previously under joint development by Takeda Chemical Industries (Osaka, Japan) and Upjohn (Kalamazoo, MI) as an oral treatment for non-insulin dependent diabetes mellitus (NIDDM) and was undergoing clinical testing for safety and efficacy. Pioglitazone hydrochloride has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues in animal models [1–3].

* Corresponding author.

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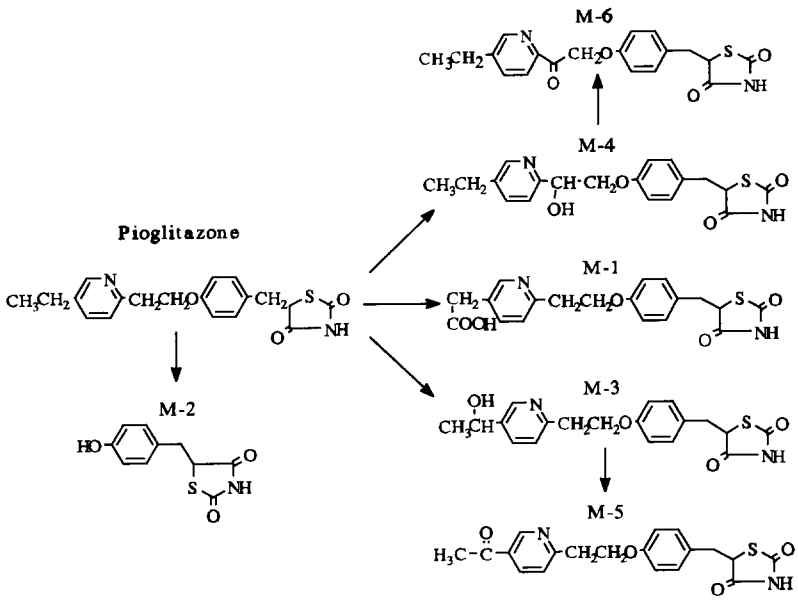

Fig. 1. Metabolic scheme of pioglitazone.

Previous pharmacokinetic studies indicated that several metabolites were present in the post-dose rat and dog serum and these have been identified as U-90441 (M-2), U-90760 (M-4), U-91322 (M-3), U-91323 (M-1), U-91324 (M-5), and U-91325 (M-6) (Fig. 1). The determination of serum concentrations of these metabolites in clinical studies would be very important for meaningful interpretation of the efficacy results since three of the metabolites, M-3, M-4, and M-5, were found to be pharmacologically active. A previously developed method for determination of pioglitazone in serum could not be used to quantitate these metabolites [4]. Although Murakami et al. of Takeda have developed a method for quantitation of pioglitazone and five of its identified metabolites in serum (unpublished method), the complicated extraction procedures, which involve solid phase extraction, liquid–liquid extraction, centrifugation, two periods of evaporation, etc., and the long chromatographic run time (60 min) with a solvent gradient program for each injection, make the method impractical for routine assay of large numbers of samples from clinical pharmacokinetic studies. Thus, an analytical method for simultaneous quantitation of the parent drug and six identified metabolites in serum using solid phase extraction (SPE) and liquid chromatography has been developed.

2. Experimental

2.1. Chemicals and reagents

Pioglitazone hydrochloride (U-72107A), U-91322, U-91323, U-91324, and U-92573 (internal standard, Fig. 2) were provided by The Upjohn Company (Kalamazoo, MI). U-90441, U-90760, and U-91325 were provided by Takeda Chemical Industries (Osaka, Japan). HPLC grade acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI). Acetic acid, ammonium hydroxide, and potassium phosphate (monobasic and dibasic) were of analytical-reagent grade and were purchased from Mallinckrodt Inc. (Paris,

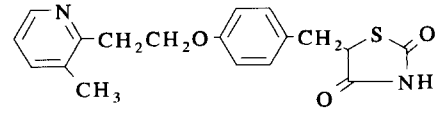

Fig. 2. Chemical structure of U-92573 (internal standard).

KY). Purified water was generated by a Milli-Q reagent water system (Millipore Corporation, Bedford, MA).

2.2. Instrumentation and chromatography

The HPLC system was composed of a Spectra-Physics SP 8810 isocratic pump with a Spectra-Physics 100 variable wavelength UV–Vis detector (San Jose, CA), and a Perkin-Elmer ISS-100 autosampler (Norwalk, CT). The chromatographic separation was accomplished on a Zorbax RX-C_8 analytical column (250 × 4.6 mm i.d., 5 μm particle, MAC-MOD Analytical Inc., Chadds Ford, PA) linked to a guard column (Rainin CN, 15 × 3.2 mm i.d.) using a mobile phase of acetonitrile–water (40:60, v/v) containing 3 ml acetic acid per liter of mobile phase with an apparent pH of 5.50 ± 0.05 adjusted with ammonium hydroxide. The chromatographic system was operated at 21–23°C with a flow rate programed from 0 to 10 min at 1.2 ml min^{-1}, and 10 to 20 min at 1.6 ml min^{-1}. The ultraviolet (UV) absorbance of column effluent was monitored at 269 nm. Quantification was accomplished by peak height ratio analysis using a structurally related compound, U-92573, as the internal standard (IS) (Fig. 2). A Harris computer system was used for calculation of the response ratio between the peak height of the drug and the IS.

2.3. Preparation of standards and controls

A drug stock standard solution was prepared by dissolving accurately weighed quantities of pioglitazone hydrochloride and each metabolite in a 25 ml volumetric flask with acetonitrile–methanol (50:50, v/v) to give a stock solution containing 100 μg ml^{-1} of free base equivalents of each compound. The IS stock solution was prepared by dissolving 2.5 mg of IS in a 25 ml volumetric flask with acetonitrile–methanol (50:50, v/v) to give a 100 μg ml^{-1} IS stock solution. The working standard solutions were prepared by diluting stock standard solution with acetonitrile–water (30:70, v/v). Stock and working standard solutions were stored at room temperature and protected from light.

Human serum standards were prepared fresh daily by aliquoting appropriate volumes of drug stock and working solutions into 0.5 ml of blank human serum (drug-free) in culture tubes to produce a concentration series ranging from 0.005 to 2 μg ml^{-1} of each compound. Reference standard solutions for the estimate of absolute recovery were prepared in acetonitrile–water (35:65, v/v).

Quality control (QC) samples were prepared by aliquoting appropriate volumes of drug stock and working standard solutions into blank human serum to yield concentrations of 0.05, 0.5, and 1.5 μg ml^{-1}. QC samples were stored at −20°C until analysis.

2.4. Processing of unknown samples

Twelve C_{18} columns (100 mg 1.0 ml^{-1}, Varian, Habor City, CA) placed on the vacuum extraction manifold (Supelco Inc., Bellefonte, PA) were prewashed with 1 column volume of acetonitrile followed by 1 column volume of 0.1 M KH_2PO_4 buffer solution. A 0.5 ml aliquot of unknown sample or serum standard was mixed with 50 μl of IS working solution (5 μg ml^{-1}) and 0.5 ml of 0.1 M KH_2PO_4 buffer solution and loaded onto individual SPE columns under slight vacuum (approximately 86 kPa). The column was rinsed with 0.3 ml methanol–0.1 M KH_2PO_4 (20:80, v/v) followed by 1 ml of 0.1 M K_2HPO_4 buffer solution and was then dried for 5 min with vacuum aspiration (approximately 27 kPa). The compounds of interest were eluted from the column with 0.5 ml of acetonitrile–water (35:65, v/v) by manually applying a slow uniform pressure to the top of the column using nitrogen gas (about 0.2 kg cm^{-2}). Each eluate was collected in a 2 ml autosampler vial. 100 μl of the eluate was injected onto the HPLC system for analysis.

2.5. Validation

Method validation was performed by analyzing freshly prepared human serum standard curves containing the seven compounds on four different days along with the low, medium, and high QC samples prepared in replicate [5–7]. The intra-assay precision was determined from the relative

standard deviations (RSDs) of QC sample concentrations analyzed on the same day and the inter-assay precision was obtained from the RSD of QC sample concentrations analyzed on four separate days over a period of 4 weeks. The assay accuracy was evaluated by comparing the actual amounts of the analytes spiked with those measured for QC samples (bias). The limit of quantitation (LOQ) was estimated by analyzing fortified serum samples at the presumed levels in replicate, at which the intra- and inter-assay precision and accuracy were acceptable (RSD or bias $\leq 20\%$). The absolute extraction recoveries were determined based on the comparison of the areas under the peaks of the extracted samples with those of unextracted reference standard solutions containing the corresponding concentrations. In all cases, the means, standard deviations (SDs), and RSDs were calculated.

3. Results and discussions

3.1. HPLC characteristics

A review of the UV responses for the parent compound and each metabolite indicated a UV maximum range of 262–275 nm. Thus, a UV wavelength of 269 nm was selected for monitoring the chromatographic effluent, giving adequate detection limits for all compounds of interest with minimal interference from endogenous serum components. By using 269 nm, several late eluting interference peaks from human serum endogenous materials (retention times ranged between 80 and 100 min) were eliminated. These interferences had quite high absorbance at 229 nm, the wavelength used in a previous method [4], and interfered with the quantitation of pioglitazone and metabolites in subsequent injections.

Initial evaluation of the HPLC characteristics of pioglitazone and its six metabolites was carried out by using the chromatographic conditions previously developed for the parent drug in serum [4]. The Jones Chromatography ODS column (250×4.6 mm i.d., 5 μm particle size) with a mobile phase of acetonitrile–water (40:60, v/v) containing 2.4 ml acetic acid per liter of mobile phase (pH 6) produced sharp symmetric peaks for pioglitazone, M-1, M-2, M-3, M-5, and M-6. However, M-4 was eluted from this column as a broad-tailed peak. Therefore, various reversed-phase analytical columns such as Phenomenex Hypersil 5 C_{18}, Phenomenex IB-Sil 5 C_{18}, Beckman Ultrasphere 5 C_{18} and Zorbax RX-C_8 were evaluated. Among them, the Zorbax RX-C_8 analytical column (250×4.6 mm i.d., 5 μm particle) gave the best peak shape for M-4. Thus, the mobile phase optimization was carried out using this column.

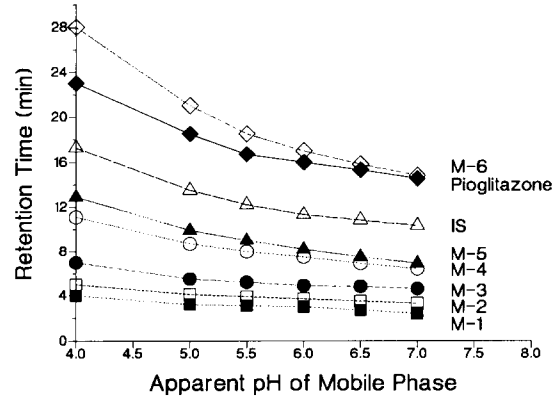

Fig. 3. Effect of mobile phase pH on the retention of pioglitazone and its metabolites.

The pH of the mobile phase was found to affect both retention time and peak shape of pioglitazone and its metabolites significantly. To select the optimal pH, a study was carried out using a mobile phase of acetonitrile–water (40:60, v/v) containing 3.0 ml acetic acid per liter mobile phase with a flow rate of 1.2 ml min^{-1}. Using a constant volume of acetic acid (3 ml l^{-1}), the apparent pH of the mobile phase was adjusted to 4, 5, 5.5, 6, 6.5 and 7 with ammonium hydroxide (40%). An increase of pH from 4 to 7 decreased the retention times of these analytes, particularly for pioglitazone, M-6, and IS (Fig. 3). A dramatic distortion of the peak shape was also observed for pioglitazone, M-6, and IS when the pH was lower than 5. However, a mobile phase with pH > 6 resulted in poor resolution between pioglitazone and M-6 and M-4 and M-5 (Fig. 3). Therefore, a mobile phase of acetonitrile–water (40:60, v/v) containing 3 ml l^{-1} acetic acid with an apparent

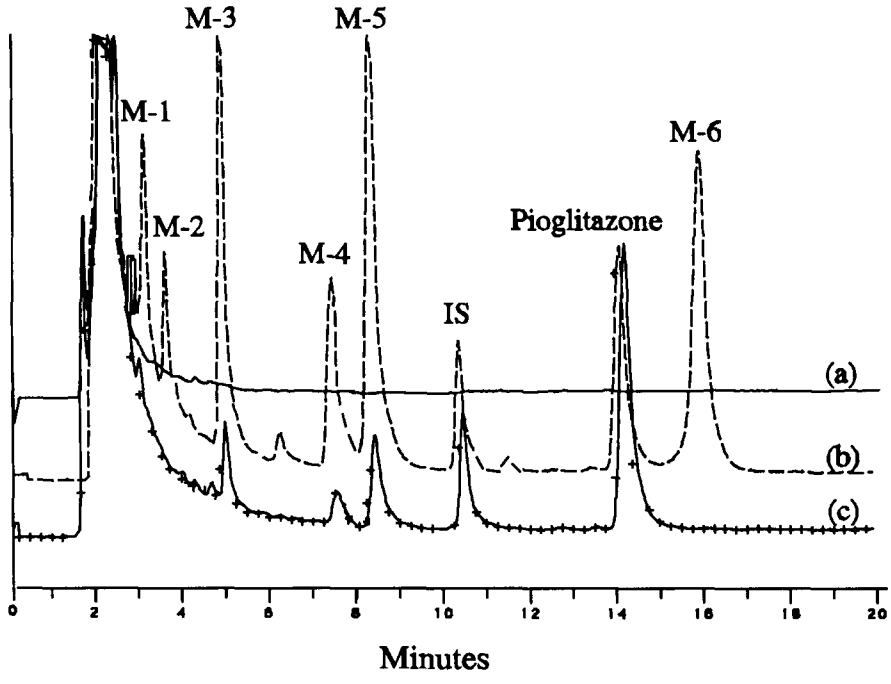

Fig. 4. Chromatograms after extraction of (a) a blank human serum, (b) a blank human serum fortified with 1 μg ml^{-1} each of pioglitazone, M-1, M-2, M-3, M-4, M-5, M-6 and IS, and (c) a human serum sample collected 2 h post-dose from subject # 13 who received a single oral dose of 20 mg pioglitazone.

pH of 5.50 $\pm$ 0.05 was selected, which gave the best separation between these analytes. A flow-rate program from 0 to 10 min at 1.2 ml min^{-1} and 10 to 20 min at 1.6 ml min^{-1} provided a chromatographic run time of 20 min for each injection, without jeopardizing the excellent resolution between the eight analytes. Representative chromatograms of drug-free human serum, serum spiked with pioglitazone, six metabolites, and IS, and a 2 h post-dose human serum sample collected from a clinical study are shown in Fig. 4.

3.2. Extraction efficiency

The extraction method previously developed for pioglitazone using C_{18} SPE [4] was first used for evaluation of extraction efficiency of pioglitazone and its six metabolites. Since most of these metabolites were much more polar than the parent drug, a modification of the SPE method was necessary to improve the extraction recoveries of these metabolites. The retention of these compounds on the SPE column increased when the serum sample was mixed with an equal volume of 0.1 M KH_2PO_4 buffer solution (pH 4.5) before loading on the SPE column. This is probably attributable to the disruption of the protein binding of these compounds by the acidic phosphate buffer. The acidic buffer may also facilitate the secondary interaction between the C_{18} sorbent and polar metabolites. The amounts of phosphate buffer mixed with the serum sample were also found to be important for the extraction efficiency. As shown in Fig. 5, mixing 0.5 ml serum sample with 0.5 ml phosphate buffer resulted in higher extraction recoveries for these analytes compared to that mixed with either 0.3 ml, 1 ml, or no phosphate buffer. The chromatogram of the extract was significantly improved with fewer interferences when the SPE column was rinsed with 300 μl of methanol–0.1 M KH_2PO_4 (20:80, v/v) after sample loading, which removed polar serum endogenous components without eluting the analytes. An elution

solvent of 0.5 ml acetonitrile–water (35:65, v/v) was the best choice with respect to the extraction recovery. Because of the secondary interaction, a column pre-condition with 1 ml of 0.1 M K_2HPO_4 buffer solution further improved the extraction recoveries of these compounds. Based on the above conditions, the recovery was approximately 93–98% for pioglitazone and M-6, 90–95% for M-4, M-3, and M-5, 60–70% for M-2, 32–36% for M-1, and 90–95% for IS. Owing to the large differences in polarity between the parent compound and metabolites, it is difficult to obtain high extraction recoveries for all of them. Thus, the emphasis was placed on the parent compound and the three active metabolites, M-3, M-4, and M-5, when optimizing the extraction conditions. With one step each for sample loading, clean-up, and elution, the extraction procedure was simple and rapid enough to allow accurate assay of large numbers of samples routinely. No apparent difference in the extraction recovery was observed when using 0.5 ml and 1 ml serum sample for extraction as long as it was mixed with an equal volume of phosphate buffer before loading on the SPE column.

3.3. Linearity

The calibration curve data for each compound in human serum during the method validation are listed in Table 1. A linear relationship was found between the peak height ratio and concentrations ranging from 0.02 to 2 $\mu g\ ml^{-1}$ for pioglitazone, M-3, M-5 and M-6; from 0.04 to 2 $\mu g\ ml^{-1}$ for M-2; and from 0.5 to 2 $\mu g\ ml^{-1}$ for M-1. Linear regression analysis indicated that the correlation coefficient (r) was greater than 0.99 with the intercept not being significantly different from zero ($p > 0.05$). The null hypothesis test also indicated that the addition of the quadratic term did not significantly ($p > 0.05$) improve the fit. Thus, the equation $Y = aX$ (force through the origin) can be used to calculate the unknown and QC samples by inverse prediction against the calibration curve for the above compounds. The relationship between peak height ratio and concentration over a range of 0.04 to 2 $\mu g\ ml^{-1}$ for M-4 was best described by a polynomial regression model of $Y = aX^2 + bX + c$ weighted by $1/X$, since the addition of the terms a and b did significanty ($p < 0.05$) improve the curve fit and the intercept was significantly different from zero. The non-linearity of the relationship for M-4 is probably due to the partial separation of stereoisomers, which has been proved by LC–MS analysis. This influenced the peak shape of M-4, especially at low concentrations.

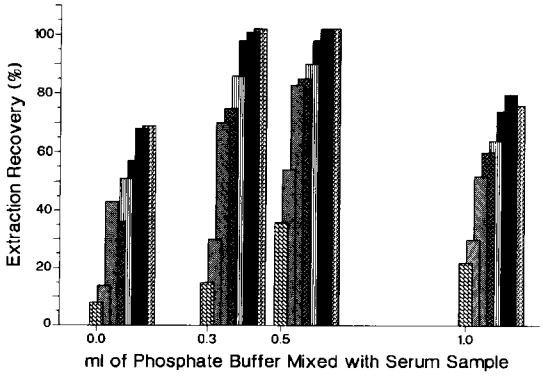

Fig. 5. The influence on extraction recoveries of the amount of phosphate buffer mixed with serum sample before loading on the SPE column (from front to back: M-1, M-2, M-3, M-4, M-5, IS, pioglitazone, and M-6).

3.4. Specificity

Specificity of this method has been demonstrated by the representative chromatograms in human serum (Fig. 4), which indicated that each analyte was well resolved from the human serum endogenous material peaks. Additional blank human serum from several individuals was also tested. There were no major interferences at the retention times of the compounds of interest. Small interferences were observed at the retention times of M-1 and M-2 and varied among individuals. As a result, the limit of quantitation for these compounds was higher than others.

3.5. Limit of quantitation

Based upon the evaluation of the replicates of low concentration standards in each validation run, the precision and accuracy values were acceptable ($\leqslant 20\%$) at concentrations of $\geqslant 0.02$ μg

Table 1
Calibration curve data for determination of pioglitazone and its metabolites in human serum

Compound	Model	Parameter	Day 1	Day 2	Day 3	Day 4
Pioglitazone	$Y=aX+b$[a]	a (RSD)	0.342 (0.6)	0.304 (0.7)	0.352 (0.6)	0.345 (0.5)
		b (SD)	2.4 (1.9)	0 (2.0)	3.3 (1.9)	0.2 (1.6)
		r	0.9998	0.9997	0.9999	0.9999
M-1	$Y=aX+b$[a]	a (RSD)	0.273 (2.7)	0.295 (1.6)	0.272 (1.4)	0.261 (2.5)
		b (SD)	−4 (7)	−5 (4)	−7 (3)	−12 (6)
		r	0.9962	0.9988	0.9991	0.9968
M-2	$Y=aX+b$[a]	a (RSD)	0.216 (3)	0.241 (1.3)	0.275 (1.8)	0.201 (2.4)
		b (SD)	3 (6)	3 (3)	3 (5)	4 (5)
		r	0.9972	0.9994	0.9990	0.9981
M-3	$Y=aX+b$[a]	a (RSD)	0.643 (1.0)	0.780 (1.3)	0.702 (1.4)	0.702 (0.4)
		b (SD)	4 (6)	0 (9)	3 (9)	2.0 (2.8)
		r	0.9995	0.9992	0.9990	0.9999
M-4	$Y=aX^b+bX^b$[b]	a (RSD)	2190 (34)	3280 (10)	3850 (23)	3430 (22)
		b (RSD)	0.237 (5.3)	0.208 (5.5)	0.277 (5.3)	0.267 (4.8)
		c (SD)	−3.1 (0.9)	−2.9 (0.9)	−3.6 (1.0)	−3.0 (0.9)
		r	0.9987	0.9992	0.9989	0.9991
M-5	$Y=aX+b$[a]	a (RSD)	0.635 (0.7)	0.685 (0.7)	0.698 (1.3)	0.697 (0.5)
		b (SD)	4 (4)	−3 (4)	1 (8)	3 (3)
		r	0.9997	0.9998	0.9992	0.9999
M-6	$Y=aX+b$[a]	a (RSD)	0.312 (0.7)	0.293 (0.9)	0.374 (1.2)	0.340 (0.9)
		b (SD)	1.2 (2.1)	−1.8 (2.5)	2 (4)	3 (5.4)
		r	0.9997	0.9996	0.9993	0.9989

[a]Data calculated with no weighting.
[b]Data calculated with weighting of $1/X$.

ml^{-1} for pioglitazone, M-3, M-5, and M-6, $\geqslant$0.04 μg ml^{-1} for M-2 and M-4, and $\geqslant$0.5 μg ml^{-1} for M-1, when using a 0.5 ml human serum sample for extraction and a 100 μl injection volume for HPLC analysis. The assay sensitivity can be increased if 1 ml of serum sample is available for extraction.

3.6. Precision and accuracy

The SPE and subsequent HPLC analysis displayed excellent intra- and inter-assay precision for pioglitazone and its metabolites. Table 2 lists the method validation results; the precision (RSD) for pioglitazone and each metabolite at the three concentrations in the within-day (intra-assay) study varied between 1.3 and 9.0% ($n=5$) and in the day-to-day (inter-assay) study varied between 3.0 and 9.0% ($n=20$). The measured concentrations of QC samples were found to be in good agreement with the actual concentrations; the bias ranged from −8.2 to 13.4% at the three concentrations evaluated for all analytes.

3.7. Applicability

The method has been used to analyze clinical serum samples. Three of the six potential metabolites, M-3, M-4, and M-5, were quantifiable in the post-dose serum samples with concentrations following the order M-3 > M-5 > M-4. As an example, the serum concentration–time profiles of pioglitazone and observed metabolites determined using the method described above are presented in

Table 2
Intra- and inter-assay precision and accuracy for determination of pioglitazone and its metabolites in human serum

Compound	Added (ng ml^{-1})	Intra-assay ($n = 5$)			Inter-assay ($n = 20$)		
		Mean (ng ml^{-1})	RSD (%)	Bias (%)	Mean (ng ml^{-1})	RSD (%)	Bias (%)
Pioglitazone	50	51.6	5.0	+3.2	49.1	6.0	−1.8
	500	520	6.0	+4.0	480	6.0	−4.0
	1500	1490	2.1	+0.7	1450	4.0	+3.3
M-1	50	<LOQ[a]	–	–	<LOQ	–	–
	500	486	5.0	−2.8	483	6.0	−3.4
	1500	1450	4.0	−3.3	1440	7.0	−4.0
M-2	50	56.7	8.7	+13.4	56.0	9.0	+12
	500	539	1.3	+7.8	500	9.0	0
	1500	1560	3.0	+4.0	1520	5.0	+1.3
M-3	50	50.9	5.0	+1.8	50.9	5.0	+1.8
	500	500	5.0	0	460	8.0	−8.0
	1500	1510	2.7	+0.6	1520	4.0	+1.3
M-4	50	45.9	4.0	−8.2	52.0	8.0	+9.0
	500	475	2.4	−5.0	505	5.0	+0.2
	1500	1500	5.0	0	1400	6.0	−6.7
M-5	50	51.8	3.0	+3.6	50.6	4.0	+1.2
	500	520	7.0	+4.0	470	8.0	−6.0
	1500	1530	3.0	+2.0	1540	4.0	+2.7
M-6	50	49.9	6.0	−0.2	51.6	5.0	+3.2
	500	510	9.0	+2.0	492	6.0	−1.6
	1500	1515	1.9	+1.0	1510	3.0	+0.7

[a]LOQ = limit of quantitation

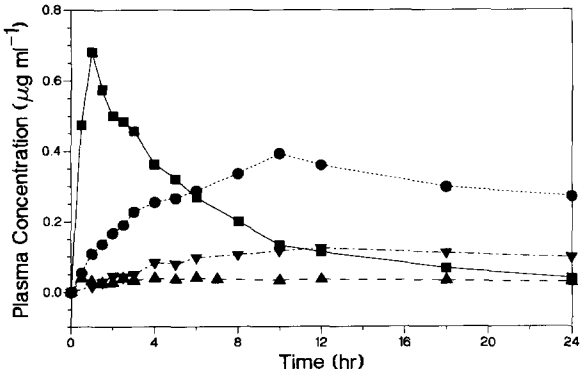

Fig. 6. Serum concentration–time profiles of pioglitazone (■), M-3 (●), M-5 (▼), and M-4 (▲) for subject # 13 who received a single oral dose of 20 mg pioglitazone.

Fig. 6 for a subject (# 13) who received a single oral dose of 20 mg of pioglitazone. The concentrations of M-1, M-2, and M-6 were all below the limit of quantitation.

4. Conclusions

The validation of this analytical method for the determination of pioglitazone and its metabolites in serum indicated excellent reproducibility. Distinct advantages include the simplicity and rapidity for sample preparation and chromatography, good resolution between the parent drug and six metabolites, accurate assay of large numbers of

samples manually and the requirement of only common instruments. The SPE procedure, with one step each for the sample loading, clean-up, and elution, can be easily automated either with a robot or an automated sample preparation system such as Zymark's BenchMate. This method, as developed, has been proved to have sufficient sensitivity to support pharmacokinetic evaluation in clinical studies.

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