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Quantitative determination of pioglitazone in human serum by direct-injection high-performance liquid chromatography mass spectrometry and its application to a bioequivalence study

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Abstract

A simple, high throughput, direct-injection high-performance liquid chromatography tandem mass spectrometry method (LC/MS/MS) has been developed and validated for the quantitation of pioglitazone in human serum. After mixing the internal standard with a sample, a 10 μl portion of the mixture was directly injected into a high-flow LC/MS/MS system, which included an extraction column, an analytical column and a six-port switching valve. The on-line extraction was achieved on an Oasis® HLB column (1 mm × 50 mm, 30 μm) with a 100% aqueous loading mobile phase containing 5 mM ammonium acetate (pH 4.0) at a flow rate of 4 ml/min. The extracted analyte was eluted by a mobile phase which contained 5 mM ammonium acetate and acetonitrile. The analytical column was a Luna C18 column (4.6 mm × 50 mm, 5 μm). Detection was achieved by positive ion electrospray tandem mass spectrometry. The lower limit of quantitation of the method was 9 ng/ml. The standard curve, which ranged from 9 to 1350 ng/ml, was fitted by a weighted ($1/x^2$) quadratic regression model. The validation results demonstrated that this method had satisfactory precision and accuracy across the calibration range. There was no evidence of instability of the analyte in human serum following three freeze-thaw cycles, and samples could be stored for at least 2 weeks at −30 °C. This method was used to analyze pioglitazone concentrations in human serum samples from a bioequivalence study of a blinded Actos® formulation (encapsulated 15 mg tablet) and an Actos® 15 mg tablet. The blinded formulation was shown to be bioequivalent to an Actos® 15 mg tablet.

Keywords: Bioequivalence study; Pioglitazone

1. Introduction

Pioglitazone hydrochloride (Fig. 1) is a thiazolidinedione antidiabetic agent used in the treatment of type 2 diabetes. Pioglitazone decreases insulin resistance in the periphery and liver, resulting in increased insulin-dependent glucose disposal and decreased

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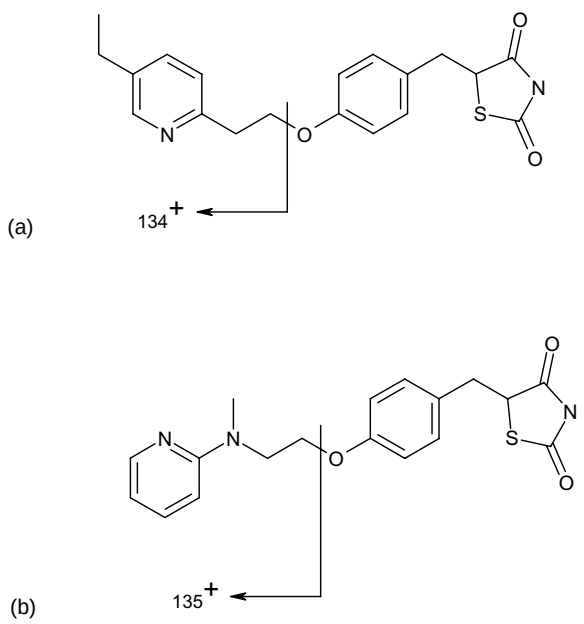


Fig. 1. Chemical structures and fragmentation of pioglitazone and the internal standard rosiglitazone. (a) Pioglitazone-free base: molecular weight, 356.5; elemental formula, $C_{19}H_{20}N_2O_3S$; monoisotopic exact mass, 356. (b) Rosiglitazone-free base: molecular weight, 357.4; elemental formula; $C_{18}H_{19}N_3O_3S$, monoisotopic exact mass, 357.

hepatic glucose output. Currently, it is marketed under the trade name Actos® [1]. An Actos® 15 mg tablet encapsulated in a gelatin capsule filled with lactose was developed for use as a blinded comparator in clinical safety and efficacy studies. A bioequivalence study of the encapsulated Actos® 15 mg tablet versus an Actos® 15 mg tablet was conducted to determine whether the rate and extent of pioglitazone absorption were altered by the blinding process.

Several methods have been reported for determining pioglitazone in biological fluids. Zhong and Lakings reported a high-performance liquid chromatographic/ultraviolet (HPLC/UV) method with a solid-phase extraction (SPE) procedure for the determination of pioglitazone in dog serum [2]. More recently, Zhong and Williams reported an analytical method for the simultaneous quantitation of pioglitazone and six identified metabolites in human serum using SPE and HPLC/UV [3]. Yamashita et al. [4] reported another HPLC/UV method for the simultaneous determination of pioglitazone and five metabolites in human serum and urine. SPE and liquid–liquid extraction (LLE) were used to purify the serum samples while LLE was used to purify the urine samples. The method was applied for the clinical trials of pioglitazone. There are two major limitations of these methods: (1) manual sample preparation (SPE or LLE) can be labor intensive and has low sample throughput and (2) conventional HPLC/UV methods have limited selectivity and specificity and usually require long run times.

Direct-injection (also called column-switch on-line SPE) has long been used as a highly automated sample preparation methodology combined with HPLC and other conventional detection modes [5–7]. However, prior to the 1990s this approach was rarely applied for analyzing biological samples by LC/MS [8–10], due to long analytical cycles ($\geq$10 min) compared with the combination of off-line cleanup techniques and LC/MS with selected reaction monitoring (SRM) [11]. With the introduction of more rugged polymer-based extraction columns in 1990s [12–19], direct-injection has become a very attractive alternative to off-line cleanup techniques because the total run time cycle can be reduced to 2–3 min.

The objective of this work was to develop a direct-injection LC/MS/MS method that could serve as a reliable high throughput method for the determination of pioglitazone in human serum. In this method, serum samples were directly injected into an extraction column for on-line sample cleanup. Subsequently, the analyte was separated by an analytical column and detected by mass spectrometry. A full validation was performed to assess the accuracy, precision, linearity, and lower limit of quantitation of the method, and the results presented here demonstrate that this method is feasible for analyzing pioglitazone in human serum. This method was subsequently used to analyze pioglitazone concentrations in human serum samples from a bioequivalence study. A summary of the pharmacokinetic results from this study is presented.

2. Experimental

2.1. Reagents and chemicals

Pioglitazone (hydrochloride salt) and the internal standard rosiglitazone (maleic acid salt) (Fig. 1) were provided by the Analytical Research and Development Department of the Bristol-Myers Squibb Pharmaceutical Research Institute. Acetonitrile (HPLC

grade) was purchased from EM Science (Gibbstown, NJ, USA) and ammonium acetate (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). In-house deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA, USA), was used. Drug-free human serum was purchased from Lampire Biological Laboratories (Pipersville, PA, USA).

2.2. Instrumentation

Chromatography was carried out using a Waters 2790 HPLC system for extraction and Shimadzu LC-10AD VP pumps for analysis. Two HPLC columns, an Oasis® HLB column (1 mm × 50 mm, 30 μm) and a Luna C18 column (4.6 mm × 50 mm, 5 μm) were used. The first column served as the sample extraction column and the second one served as the analytical column. The connections of the Oasis® extraction column to the six-port switching valve, the analytical column and the mass spectrometer are shown in Fig. 2.

A Micromass Quattro LC/MS/MS system (Beverly, MA, USA) operating under MassLynx® 3.4 software was used. The electrospray ion source was run in a positive ionization mode for all experiments. The ion-source parameters were: capillary 1.0 kV; cone 25 V; RF lens 0.15 V; source temperature 100 °C; desolvation temperature 300 °C; nebuliser 73 l/h; and drying gas 893 l/h. Quadrupole 1 (Q1) parameters were: low mass resolution 15 V; high mass resolution 15 V; and ion energy 1 V. Quadrupole 2 (Q2) parameters were: collision gas 2.5 e-3 mbar; and collision energy 25 V. Quadrupole 3 (Q3) parameters were: low mass resolution 15 V; high mass resolution 15 V; and ion energy 2 V. The multiplier was set at 650 V. The samples were analyzed via SRM. The monitoring ions were set from *m/z* 357 to 134 for pioglitazone and from *m/z* 358 to 135 for the IS. The scan dwell time was set 0.2 s for both channels.

2.3. Procedure

A 10-μl portion of the processed human serum standard or QC sample was injected by the autosampler into an Oasis® column, using 5 mM ammonium acetate in water (mobile phase A) at a flow rate of 4.0 ml/min, with the effluent directed to waste (Fig. 2a). This sample extraction stage lasted for 0.3 min. The valve was then switched so that the Oasis® column was in-line with the analytical column and the mass spectrometer, with 50% of 10 mM ammonium acetate in 10% acetonitrile/90% water (mobile phase B) and 50% of 10% water/90% acetonitrile (mobile phase C) at a flow rate of 1.35 ml/min (Fig. 2b). At this elution stage, the analyte and internal standard were eluted from the Oasis® column onto the analytical column. The effluent from the analytical column was split with a Valco fix splitter, and the split ratio was defined by the length and internal diameter of the tubing. Approximately, 40% of the effluent entered the mass spectrometer (∼0.5 ml/min), and the rest went to a waste container. After 1.0 min, the valve was switched so that the Oasis® column was first washed with 100% mobile phase C, and then conditioned as done in the extraction stage using mobile phase A at a flow rate of 4.0 ml/min (Fig. 2a). The total run time was 2.5 min. The retention times for pioglitazone and the IS were approximately 1.9 and 1.2 min, respectively.

2.4. Standard, QC and IS preparations

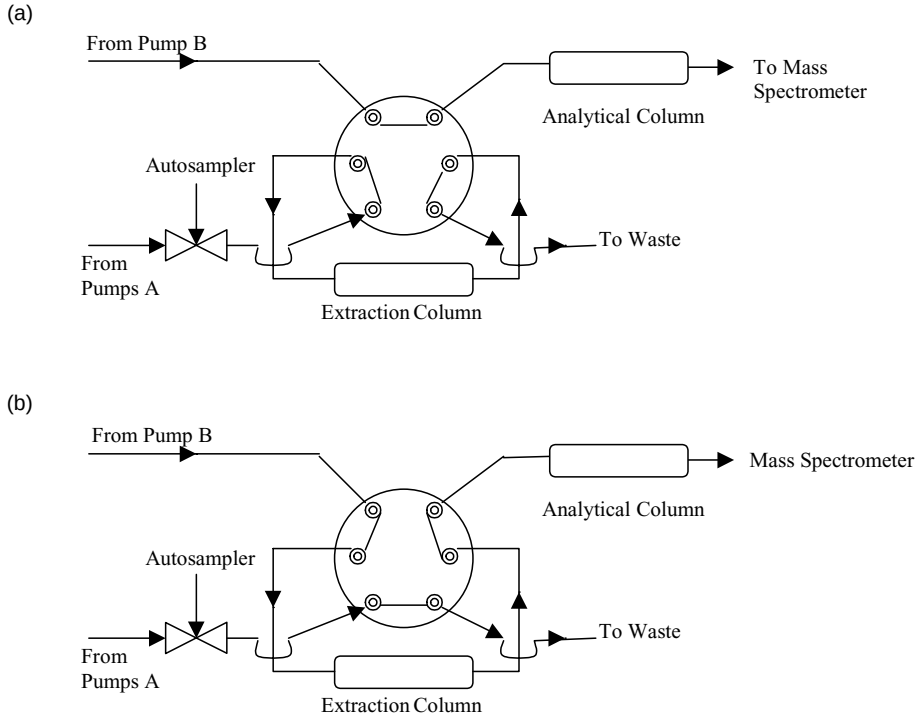
A 900 μg/ml standard stock solution was prepared by weighing the appropriate amount of pioglitazone and dissolving it into dimethylsulfoxide (DMSO). This stock solution was diluted 1:9 with DMSO to obtain a 90 μg/ml stock solution. A 100 μl portion of the 90 μg/ml stock solution was diluted to 2 ml with control human serum to yield a 4500 ng/ml stock solution. Calibration standards were prepared with each extraction set from the 4500 ng/ml stock solution by diluting with control human serum. The final standard concentrations in human serum were 1350, 1125, 900, 450, 180, 90, 54, 18 and 9 ng/ml.

A 900 μg/ml quality control (QC) stock solution was prepared from a separate weighing of pioglitazone. Dilutions were used to prepare four levels of QCs in human serum, 9000 (dilution QC), 1080, 225 and 27 ng/ml. The QC samples were stored at −30 °C.

A 750 μg/ml stock solution of rosiglitazone was prepared in DMSO and subsequently diluted with water to obtain a 375 ng/ml working internal standard solution.

2.5. Sample processing procedure

A 0.1 ml portion of the IS working solution was added to 0.1 ml of each calibration standard and QC



Time	Pump A Mobile Phase		Configuration	On-line extraction stage
	A	C		
0.0	100	0	(a)	Extraction
0.3	100	0	(b)	Elution
1.0	0	100	(a)	Cleanup extraction column
2.0	100	0	(a)	Equilibrate extraction column

Pump A: 5 mM ammonium acetate in water (mobile phase A) and 10% water/90% ACN (mobile phase C).

Fig. 2. A schematic representation of the on-line extraction LC/MS/MS system. (a) Loading sample and extraction or cleanup and equilibrium. (b) Elution.

sample in an LC vial. Dilution QC samples were diluted 1:9 with control human serum and then 0.1 ml of the diluted sample was used for analysis. The samples were vortexed for 1.0 min and centrifuged for 10 min prior to injection.

2.6. Bioequivalence study

2.6.1. Study design

The study was conducted as an open-label, randomized, two-period, two-treatment crossover study. Twenty-eight healthy male subjects received one encapsulated Actos® 15 mg tablet or one Actos® 15 mg tablet in one of two randomly assigned treatment sequences. Each treatment period was separated by at least a 1-week washout period.

2.6.2. Sample collection and analysis

Serial blood samples (7 ml per sample) were collected from an indwelling catheter or by direct venipuncture pre-dose, and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 16, 24, and 32 h post-dose. After collection, blood samples remained at room temperature for 30 min to allow the blood to clot. Immediately

afterwards, blood samples were centrifuged for 15 min at approximately 1000 × *g* and 4 °C. Serum was harvested, transferred to screw-capped polypropylene tubes, and stored frozen at −30 °C until analysis.

2.6.3. Pharmacokinetic analysis

Individual subject pioglitazone serum concentration versus time profiles were analyzed by non-compartmental analysis [20]. The maximum observed serum concentration (C_{max}) and the time of maximum observed serum concentration (T_{max}) were recorded directly from experimental observations. Pioglitazone area under the curve from time zero until the time of the last quantifiable concentration [AUC(0–*T*)] was calculated by the linear trapezoidal rule. The terminal elimination half-life and area under the curve from time zero extrapolated to infinite time [AUC(INF)] were not reported since several subjects had AUC(0–*T*)/AUC(INF) values less than 0.85.

2.6.4. Statistical analysis

To determine bioequivalence of the encapsulated Actos® 15 mg tablet relative to the Actos® 15 mg tablet, analyses of variance were performed on log(C_{max}) and log[AUC(0–*T*)]. Since subjects were random effects nested within sequences, *F*-statistics for sequence effects were the ratios of the type I mean squares for sequence and subjects within sequence. The *F*-statistic for period was the ratio of the type I mean square for period and the mean square for error. Point estimates and 90% confidence intervals for means and differences between means on the log scale were exponentiated to obtain estimates for geometric means and ratios of geometric means on the original scale. No adjustment was made for multiplicity. According to the current FDA Guidances for Industry [21,22], bioequivalence was to be concluded if the 90% confidence intervals for the ratios of population geometric means for the encapsulated Actos® 15 mg tablet relative to the Actos® 15 mg tablet were entirely contained between 80 and 125% for both C_{max} and AUC(0–*T*).

2.6.5. Safety assessments

Safety assessments were based on the medical review of adverse event reports, and the results of vital sign measurements, physical examinations, and clinical laboratory tests. Adverse events were obtained by questioning and monitoring of the subjects, and through information volunteered by the subjects.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample preparation

During method development, human serum samples initially were diluted 1:1 (v/v) with acetonitrile containing the IS. However, the addition of this percentage of acetonitrile resulted in poor reproducibility due to incomplete precipitation of plasma proteins. Adding the IS in a purely aqueous media generated consistent results. Aqueous dilution of biological samples avoids analyte loss by maintaining its binding to proteins prior to injection onto the extraction column. This is consistent with one report [18] that suggested organic solvents should be not more than 20% of the plasma volume, even with poorly water-soluble compounds.

3.1.2. Direct-injection procedure

3.1.2.1. Selection of extraction column washing solvents. During method development, 10 mM ammonium acetate in water/acetonitrile (1:9) was initially used to wash the extraction column during the equilibration stage; however, a relatively high carryover (∼0.5%) was observed for both the analyte and the IS. To reduce the carryover effects, 10 mM ammonium acetate was omitted from the solvent combination. With water/acetonitrile, the carryover observed was between 0.05 and 0.1% for the highest standard, which translated to less than 15% of the lowest standard concentration, 9 ng/ml. The low level of the carryover allowed validation of this method with a broader calibration range (9–1350 ng/ml). Appropriate selection of organic solvents is, therefore, critical in reducing or eliminating the carryover caused by the analyte adhering to many parts of the system, including the autoinjector, tubing, valve and the extraction column [12,16,18].

3.1.2.2. Selection of analysis column. Among the C18 columns screened, Waters Xterra MS C18 had the best peak shape (more symmetrical and less tailing) and most consistent retention times for both the

analyte and the IS. However, during method validation, the Xterra column was observed to have a very short column life, and this was considered unsuitable for routine sample analysis. The peak shape started to deteriorate after 80 injections, along with an increase in column back-pressure. The most likely cause of degraded performance was retention of biological materials on the stationary phase. To improve method ruggedness, the Waters Xterra column was replaced with a Phenomenex Luna column. Although the analyte and IS peak shape from the Luna column were not as good as that achieved with the Xterra column, the Luna column had much longer column life. On an average, the number of injections for a column was 600. When a 0.2 μm on-line filter was used, the column life was extended further. The filter was changed on a regular basis (approximately every 300 samples). The replacement of the filter was easy and it took less than 1 min. As a comparison, under the separation conditions used, both columns separated pioglitazone from its IS. Chromatographic separation was critical to avoid crosstalk, since they differed by only one mass unit. Both (Xterra MS C18 4.6 mm × 50 mm, 5 μm; Luna C18 4.6 mm × 50 mm, 5 μm) columns exhibited similar plate numbers (∼5000), and the signal to noise ratios obtained from lower limit of quatitation (LLOQ) samples were equivalent. Therefore, these two columns behaved almost identically, except for the tolerance levels to biological materials.

3.1.2.3. Improved band broadening. At a high flow rate (4.0 ml/min), the mobile phase flow conditions are turbulent rather than laminar. This results in a solvent front profile which resembles a step rather than a parabolic function. Thus, it is expected that band broadening from the high flow rate would be minimal [12,18]. Band broadening was clearly evident with a much wider peak width when a low flow rate (such as 0.2 ml/min) was used for the analytical column. Our preliminary experiments showed that a higher flow rate for the analytical column was required to compensate for the band broadening. Most applications have used higher analytical flow rates than normally used with MS detection due to difficulties in re-focusing the extracted sample [13,14,16]. The optimized flow rate for the analytical column used in this validation was 1.35 ml/min. Due to the high flow rate, a standard 4.6 mm diameter column was used to reduce excessive high back-pressure. This ion source would not have been able to handle a 1.35 ml/min flow rate, and therefore, flow splitting was required to avoid flooding the ion-source chamber.

3.1.2.4. Detection sensitivity. The LLOQ for this method was 9 ng/ml. It could be lowered further by increasing the sample injection volume from 10 μl to as high as 200 μl. The use of higher injection volumes to achieve lower limits of quantitation has been reported by others [12,18]. However, by utilizing this approach, the lifetime of the extraction and analytical columns may be reduced. In addition, method sensitivity may not increase proportionally due to the peak broadening associated with the larger injection volumes.

Although the six human pioglitazone metabolites were not available and therefore not monitored, their potential interference to the analyte peak was investigated by analysis of incurred dog serum samples. The two metabolites, M3 (alcohol) and M5 (aldehyde) observed in these samples, had different retention times and SRM transitions. The remaining four metabolites were predicted to be at trace levels in human serum, and their SRM transitions were expected to be different from the parent [3,4]. Therefore, it was concluded that these metabolites would not interfere with measuring the parent compound.

3.1.3. Electrospray ionization tandem mass spectrometry

Electrospray positive MS spectra for both compounds were dominated by the $[M + H]^+$ ions: *m/z* 357 for pioglitazone and *m/z* 358 for IS. The MS/MS product ion spectra of the $[M + H]^+$ for pioglitazone and rosiglitazone produced major product ions at *m/z* 134 and 135, respectively. Fig. 1 illustrates the fragmentation of each compound. Thus, the SRM used ranged from *m/z* 357 to 134 for pioglitazone and from *m/z* 358 to 135 for IS. One precaution was that pioglitazone contained a pyridine moiety substituted in position 1 with an aliphatic group, while rosiglitazone contained the same pyridine moiety substituted with amine functionality. The ionization efficiency differences observed between the analyte and its internal standard were probably due to the substitution of the pyridine ring in position 2 with nitrogen rather than carbon and the difference in the

electron donating abilities of the substituents. Therefore, ion-source parameters, especially the capillary voltage and the source temperature, were tuned so that any minor variations of the parameters did not significantly affect the ionization.

3.2. Method validation

3.2.1. Standard curves

After the direct-injection chromatography procedure and the MS/MS conditions were defined, a full validation was performed to assess the performance of the method. A 9-point calibration standard curve ranging from 9 to 1350 ng/ml of pioglitazone in human serum was used in duplicate in each analytical run. Peak area ratios of pioglitazone to IS were used for regression analysis. A linear regression model was evaluated first; however, a weighted ($1/x^2$) quadratic regression model, where x is the concentration of pioglitazone, provided a better fit for the validation data at the higher end of the calibration curve due to non-linear response from mass spectrometry. Therefore, the weighted ($1/x^2$) quadratic regression model was used for this validation. Table 1 shows the summary of the individual standard curve data obtained in the four runs used to determine the accuracy and precision of the method and assess sample stability. The back-calculated concentrations of the 72 standards in the four analytical runs deviated not more than 13.6% from nominal concentrations. The regression coefficients (R^2) for the four runs were greater than 0.99 (data not shown). Based on the standard data presented here, it was concluded that the calibration curves used in this method were precise and accurate for the measurement of pioglitazone in human serum.

3.2.2. Accuracy and precision

The accuracy and precision of the method was assessed by analyzing QC samples at concentrations within the lower, second, and upper quartiles of the standard curve. A fourth QC sample, with a concentration higher than the upper limit of the standard curve range, was also analyzed. This QC sample, known as the dilution QC, was diluted 1:9 with control human serum prior to processing and analysis. Five replicate samples at each concentration were analyzed in three separate runs. Accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values. The intra- and inter-assay precision was expressed as percent coefficient of variation (%CV).

Table 1
Individual standard curve concentration data for pioglitazone in human serum

Nominal concentration (ng/ml)	Run #1		Run #2		Run #3		Run #4	
	Concentration (ng/ml)	Deviation (%)	Concentration (ng/ml)	Deviation (%)	Concentration (ng/ml)	Deviation (%)	Concentration (ng/ml)	Deviation (%)
9	9.34	3.7	9.40	4.5	8.26	−8.2	9	0.0
9	8.97	−0.3	8.43	−6.3	9.79	8.8	9.20	2.3
18.0	15.55	−13.6	19.28	7.1	17.57	−2.4	17.57	−2.4
18.0	19.15	6.4	17.08	−5.1	18.08	0.4	17.01	−5.5
54.0	53.44	−1.0	58.09	7.6	50.94	−5.7	56.84	5.3
54.0	56.44	4.5	55.58	2.9	58.71	8.7	58.78	8.9
90.0	79.51	−11.7	85.25	−5.3	85.23	−5.3	86.30	−4.1
90.0	91.88	2.1	84.90	−5.7	90.90	1.0	89.43	−0.6
180	176.79	−1.8	189.30	5.2	185.25	2.9	175.79	−2.3
180	201.18	11.8	179.72	−0.2	193.41	7.5	176.07	−2.2
450	406.61	−9.6	444.80	−1.2	425.41	−5.5	450.05	0.0
450	509.47	13.2	444.40	−1.2	430.10	−4.4	470.23	4.5
900	826.84	−8.1	914.39	1.6	908.59	1.0	818.17	−9.1
900	915.54	1.7	851.69	−5.4	867.94	−3.6	935.63	4.0
1125	1012.21	−10.0	1075.46	−4.4	1210.84	7.6	1158.05	2.9
1125	1271.25	13.0	1063.47	−5.5	1094.16	−2.7	1033.51	−8.1
1350	1292.69	−4.2	1512.02	12	1405.75	4.1	1347.94	−0.2
1350	1407.13	4.2	1341.80	−0.6	1294.20	−4.1	1440.75	6.7

Table 2
Individual quality control sample concentration data for pioglitazone in human serum

Nominal concentration (ng/ml)	Run #1		Run #2		Run #3		Run #4	
	Concentration (ng/ml)	Deviation (%)	Concentration (ng/ml)	Deviation (%)	Concentration (ng/ml)	Deviation (%)	Concentration (ng/ml)	Deviation (%)
27.0	28.14	4.2	28.01	3.7	26.60	−1.5	27.99	3.7
27.0	28.21	4.5	31.01	14.9	29.44	9.0	31.71	17.5
27.0	25.22	−6.6	28.50	5.6	29.81	10.4	26.93	−0.3
27.0	28.58	5.8	29.25	8.3	28.72	6.4	30.52	13.0
27.0	30.13	11.6	27.67	2.5	27.78	2.9	32.62	20.8
225	217.48	−3.3	222.80	−1.0	222.68	−1.0	254.48	13.1
225	192.72	−14.3	214.68	−4.6	222.03	−1.3	236.76	5.2
225	216.88	−3.6	220.81	−1.9	222.82	−1.0	238.16	5.8
225	205.31	−8.8	215.08	−4.4	222.50	−1.1	251.05	11.6
225	216.19	−3.9	205.21	−8.8	228.33	1.5	243.03	8.0
1080	1055.22	−2.3	1026.25	−5.0	991.54	−8.2	1028.97	−4.7
1080	986.00	−8.7	1174.12	8.7	1070.33	−0.9	1062.24	−1.6
1080	991.24	−8.2	1007.68	−6.7	1119.12	3.6	1125.52	4.2
1080	1105.33	2.3	998.17	−7.6	997.18	−7.7	1124.45	4.1
1080	1021.87	−5.4	960.69	−11	922.99	−14.5	1065.32	−1.4
9000	8669.29	−3.7	9182.97	2.0	8281.26	−8.0	9523.66	5.8
9000	8231.90	−8.5	8434.26	−6.3	8565.48	−4.8	9068.18	0.8
9000	8242.95	−8.4	8760.81	−2.7	8567.98	−4.8	8922.49	−0.9
9000	8692.17	−3.4	8747.49	−2.8	8294.52	−7.8	8885.01	−1.3
9000	8612.24	−4.3	8294.46	−7.8	7525.19	−16.4	9368.90	4.1

Table 2 shows the summary of the individual QC data obtained in the four runs used to validate the method. In each run, the deviations of the predicted concentrations from their nominal values were within ±15% for at least 95% of the QC samples. To further assess accuracy and precision, a one-way ANOVA was performed for the first three runs, and the results are shown in Table 3. The intra-assay precision was within 6.9% CV and the inter-assay precision was within 2.8% CV. The assay accuracy was within ±5.8% of the nominal values. Since QC samples are representative of study samples, similar precision and accuracy are expected from study samples. The QC data indicated that the direct-injection LC/MS/MS method was accurate and precise in the determination of pioglitazone concentrations in human serum.

Table 3
Accuracy and precision for the pioglitazone method in human serum

	Nominal concentration (ng/ml)			
	27.0	225	1080	9000
Mean observed concentration	28.5	216	1029	8474
Deviation (%)	5.4	−3.8	−4.8	−5.8
Inter-assay precision (%CV)	0.0	2.8	0.0	1.8
Intra-assay precision (%CV)	5.2	3.5	6.9	4.1
Total variation (%CV)	4.9	4.5	6.2	4.5
N	15	15	15	15
Number of runs	3	3	3	3

3.2.3. Lower limit of quantitation

To establish the LLOQ, six different lots (#1–6) of control human serum were spiked at 9 ng/ml to obtain six LLOQ samples. The LLOQ samples were processed and analyzed with a standard curve and QC samples, and their predicted concentrations determined. The results of the LLOQ determinations at 9 ng/ml are shown in Table 4. The deviations of the predicted concentrations for all six LLOQ samples were within ±18.6% of the nominal value. A typical SRM chromatogram at the LLOQ is shown in Fig. 3. In addition, 18 LLOQ samples from a single lot (#7) were analyzed. Their deviations (≤15.5%) were very similar to what was obtained from six individual lots. This suggested that endogenous materials present in

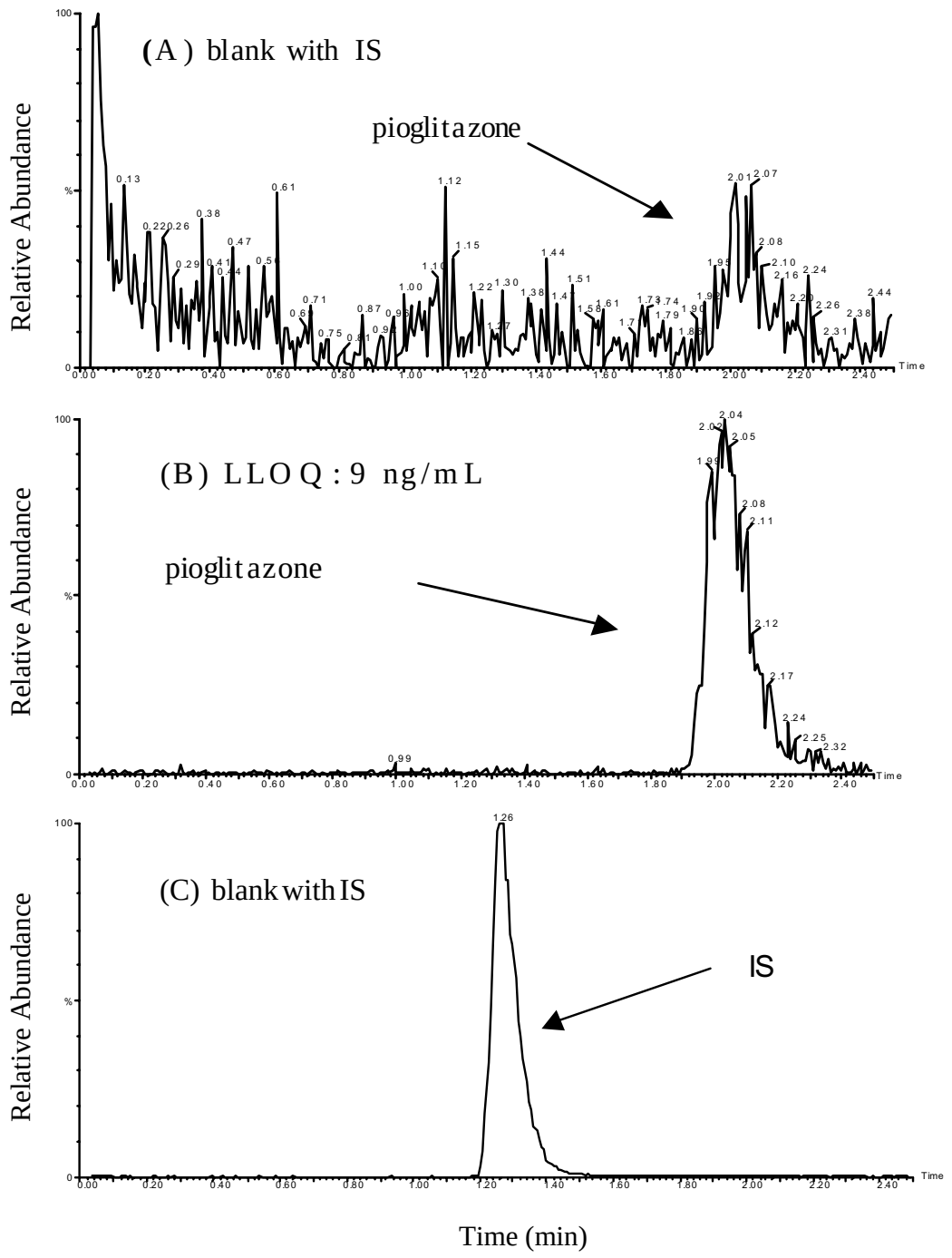


Fig. 3. Selected reaction monitoring chromatograms for pioglitazone obtained from (A) human serum containing only IS at 375 ng/ml; (B) human serum containing pioglitazone at the LLOQ (9 ng/ml) and its IS at 375 ng/ml; and (C) IS channel.

Table 4
Lower limit of quantitation (9 ng/ml) determination of pioglitazone in human serum

Lot #	Predicted concentration (ng/ml)	Deviation (%)	Mean concentration (ng/ml)	Mean deviation (%)
1	8.07	−10.4	8.34	−7.3
2	10.07	12.0		
3	7.32	−18.6		
4	7.75	−13.9		
5	8.48	−5.8		
6	8.35	−7.2		
7	9.81	9.0	8.96	−0.4
7	7.74	−14.0		
7	9.36	4.0		
7	8.82	−2.0		
7	8.54	−5.1		
7	9.16	1.8		
7	9.69	7.7		
7	8.55	−5.0		
7	10.40	15.5		
7	7.85	−12.8		
7	10.07	11.9		
7	7.73	−14.2		
7	8.23	−8.6		
7	9.96	10.7		
7	9.80	8.9		
7	7.98	−11.4		
7	9.86	9.6		
7	7.74	−14.0		

varying amounts in different serum lots were effectively removed on the extraction column. In addition, the void time of the LC system was around 0.7 min (including 0.3 min for on-column extraction), but the analyte and its internal standard were eluted later with retention times 1.9 and 1.2 min. With the combination of on-line extraction and chromatographic separation, the matrix effect should be minimal [23–27]. Furthermore, the deviations from both the individual lots and the single lot were within the acceptance criterion (≤20%) for the LLOQ samples recommended by the FDA Guidance [28]. Given the fact that the analyte and its internal standard were structurally similar but differed in their proton affinity, the LLOQ data obtained were acceptable.

3.2.4. Specificity

Six different lots of control human serum were analyzed with and without IS to determine whether any endogenous serum constituents interfered with the analyte or the IS. The degree of interference was assessed by inspection of SRM chromatograms. No significant interfering peaks from the serum were found at the retention time and in the ion channel of either the analyte or the IS. Fig. 3A and B illustrate chromatograms of blank serum.

3.2.5. Stability

The stability of pioglitazone in human serum was investigated at room temperature and under storage conditions (−30 °C). Serum samples at two pioglitazone concentrations (27 and 1080 ng/ml) were used for the stability experiments. In addition, pioglitazone stability was investigated in fresh human blood at concentrations of 29.4 and 924 ng/ml. In serum, pioglitazone was found to be stable for at least 2 weeks at −30 °C, for 48 h at room temperature, and for three freeze-thaw cycles. Samples spiked with IS were stable for at least 96 h at room temperature. When spiked into fresh human blood, pioglitazone was stable for at least 2 h at room temperature and for at least 2 h on wet ice.

3.3. Bioequivalence study results

A total of 28 subjects were enrolled, randomized, and treated. Two subjects did not complete the study: one subject did not return for Period 2 dosing and was considered lost to follow-up; the other subject was discontinued from the study prior to Period 2 due to an adverse event unrelated to study medication. The data from these subjects were not included in the statistical analyses of pioglitazone pharmacokinetic parameters.

A summary of pioglitazone pharmacokinetic parameters and the results of statistical analyses are presented in Table 5. Mean pioglitazone serum concentration versus time profiles are shown in Fig. 4. The 90% confidence intervals for pioglitazone C_{max} and AUC(0–T) geometric mean ratios were between 0.80 and 1.25, indicating that the encapsulated Actos® 15 mg tablet was bioequivalent to the Actos® 15 mg tablet. Pioglitazone median T_{max} was similar for the encapsulated Actos® 15 mg tablet (2.0 h) and the Actos® 15 mg tablet (2.3 h).

No serious adverse events were reported in this study. All adverse events were rated as either mild or moderate in intensity by the investigator and resolved prior to study discharge or upon follow-up. Single oral

Table 5
Summary of pioglitazone pharmacokinetic parameters and the results of statistical analyses

Parameter	Adjusted geometric means		Ratio of geometric means	
	Tablet	Encapsulated tablet	Point estimate	90% CI
C_{max} (ng/ml)	524	506	0.96	0.87, 1.06
AUC(0–T) (ng·h/ml)	4590	4448	0.97	0.91, 1.03
T_{max} (h)[a]	2.3 (0.5, 4.0)	2.0 (1.0, 5.0)	–	–

[a] Median (minimum, maximum).

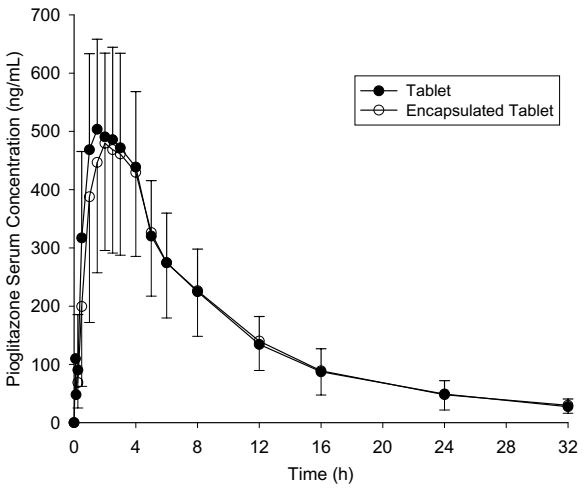


Fig. 4. Mean (S.D.) pioglitazone serum concentration vs. time profiles in healthy male subjects. Tablet: $N = 26$ subjects; encapsulated tablet: $N = 26$ subjects.

doses of pioglitazone were considered to be safe and well tolerated in this study.

4. Conclusions

This direct-injection LC/MS/MS method provides a sensitive and reliable procedure for the determination of pioglitazone in human serum. The on-line extraction and cleanup procedure of human serum resulted in a method with very short sample preparation time, high sample throughput, and good accuracy and precision. Thus, this method is suitable for routine sample analysis. The method was successfully used to analyze pioglitazone concentrations in human serum samples from a bioequivalence study. The results from the bioequivalence study indicated that the encapsulated Actos® 15 mg tablet was bioequivalent to an Actos® 15 mg tablet.

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