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A 96-well single-pot protein precipitation, liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the determination of muraglitazar, a novel diabetes drug, in human plasma

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Abstract

A 96-well single-pot protein precipitation, liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the determination of muraglitazar, a PPAR α/γ dual agonist, in human plasma. The internal standard, a chemical analogue, was dissolved in acetonitrile containing 0.1% formic acid. The solvent system was also served as a protein precipitation reagent. Human plasma samples (0.1 mL) and the internal standard solution (0.3 mL) were added to a 96-well plate. The plate was vortexed for 1 min and centrifuged for 5 min. Then the supernatant layers were directly injected into the LC/MS/MS system. The chromatographic separation was achieved isocratically on a Phenomenox C18(2) Luna column (2 mm × 50 mm, 5 μm). The mobile phase contained 20/80 (v/v) of water and acetonitrile containing 0.1% formic acid. Detection was by positive ion electrospray tandem mass spectrometry on a Sciex API 3000. The standard curve, which ranged from 1 to 1000 ng/mL, was fitted to a $1/x$ weighted quadratic regression model. This single-pot approach effectively eliminated three time consuming sample preparation steps: sample transfer, dry-down, and reconstitution before the injection, while it preserved all the benefits of the traditional protein precipitation. By properly adjusting the autosampler needle offset level, only the supernatant was injected, without disturbing the precipitated proteins in the bottom. As a result, the quality of chromatography and column life were not compromised. After more than 600 injections, there was only slightly increase of column backpressure. The validation results demonstrated that this method was rugged and provide satisfactory precision and accuracy. The method has been successfully applied to analyze human plasma samples in support of a first-in-man study. This method has also been validated in monkey and mouse plasma for the determination of muraglitazar.

Keywords: Single-pot protein precipitation; LC/MS/MS; Muraglitazar

1. Introduction

Muraglitazar (Fig. 1) is a novel oxybenzylycine analog that shows potent and balanced agonist activity at both α and γ peroxisome proliferator-acitived receptor (PPAR) isoforms. Activation of PPARα (expressed mainly in the liver) results in decreased circulating triglyceride levels and increased HDL cholesterol levels in humans. Activation of PPARγ (expressed predominantly in fat cells) results in improved insulin sensitivity and glucose utilization. PPARα agonists, such as gemfibrozil [1], are currently in use for the treatment of hypertriglyceridemia, while PPARγ agonists, such as pioglitazone [2] and rosiglitazone [2], are used to treat hyperglycemia and insulin resistance in Type 2 diabetic patients [3]. Therefore, dual agonism of PPARα and PPARγ is a novel monotherapeutic approach for the treatment of Type 2 diabetes and the often-associated dyslipidemia [4]. Currently, this compound is being developed for the treatment of type-2 diabetes [5].

LC/MS/MS has become the method of choice for drug analysis in biological fluids because of its inherent sensitivity, high specificity and speed, which have made the sample preparation the rate-limiting step [6]. Among different sample preparation techniques, protein precipitation has been widely used because of its speed, simplicity and wide applicability [7–23]. Polson et al. [7] evaluated various protein precipitants by examining their effectiveness at plasma protein removal and the extent of ionization suppression (matrix effect) in LC/MS/MS. They concluded that the optimal bioanalytical methodologies in positive ion tur-

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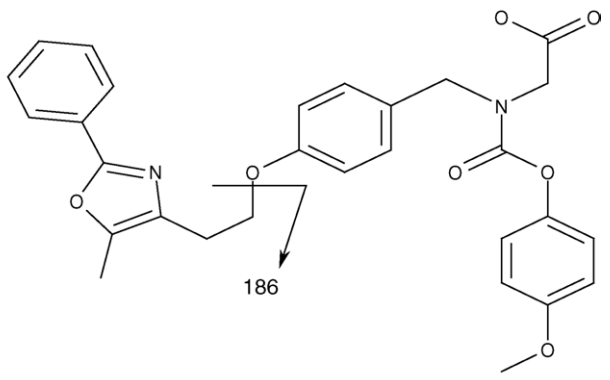

muraglitazar
$C_{29}H_{28}N_2O_7$
Molecular weight: 516.6
Monoisotopic exact mass: 516.2

Fig. 1. Chemical structure of muraglitazar.

boionspray LC/MS/MS involve either the use of trichloroacetic acid for precipitation with mobile phase consisting of pure organic solvents (methanol/water or acetonitrile/water) or precipitation with any of the mass spectrometer compatible precipitants with a methanol/aqueous 0.1% formic acid mobile phase. This technology has been widely used for measuring systemic exposures for drug candidates or other compounds, such as ribavirin in human, rat, and monkey plasma [8,9], levovirin in rat and monkey plasma [10], dioscin in rat plasma [11], and cefixime in human plasma [12].

The modern robotic technology in combination with 96-well plate has made the traditional protein precipitation sample preparation technique much more efficient. Watt reported an automated 96-well protein precipitation, LC/MS/MS method in a drug discovery environment. The validation studies revealed that the application of robotics to sample preparation maintained the analytical accuracy and precision compared to manual samples preparation [13]. Biddlecombe and Pleasance reported protein precipitation by filtration in 96-well format for plasma sample preparation, and demonstrated it can be a viable alternative to conventional protein precipitation. The use of 96-well filtration plates eliminates the need for centrifugation [14]. Walter et al. compared manual protein precipitation versus 96-well filter plate protein precipitation in term of accuracy, precision, reproducibility and linearity. They concluded that both approaches provided comparable accuracy, precision, reproducibility and linearity results, but in overall, the mean data appeared to be more reproducible for the filtration method [15]. This approach has also been applied to highly protein-bound compounds in plasma with equally successful outcomes [16,17].

However, all above applications for protein precipitation required the supernatants to be aliquoted into a separate 96-well plate or vials, then followed by the dry-down and reconstitution steps before injection into the LC/MS system. Bakhiar et al. first reported a method for the quantification of the anti-leukemia drug ST571 and its main metabolite CGP 74588 in human plasma using semi-automated protein precipitation method and a relative rapid LC/APCI/MS/MS analysis [18], where the supernatants were transferred to a clean 96-well block, and then directly injected into the LC/MS/MS system without drying down. Frigerio et al. went further by directly injecting the supernatants into an LC/MS/MS system without aliquoting to a separate 96-well plate for the quantitation of PNU-248686A in human plasma [19]. The same approach was used for the determination of tobramycin in serum [20], tetramethylpyrazine and its metabolite in dog plasma [21], and metformin in plasma samples [22]. More recently, Johannessen reported the simultaneous determination of pyrimethamine, sulfadiazine and *N*-acetyl-sulfadiazine in plasma by direct injection of the protein precipitation supernatants to an LC/MS system [23].

The objective of this work was to develop a 96-well single-pot protein precipitation, LC/MS/MS method that could serve as a reliable high throughput method for the determination of muraglitazar, a novel diabetes drug, in human plasma. In this method, human plasma samples (0.1 mL) and acetonitrile with 0.1% formic acid (0.3 mL) were added to a 96-well plate. The plate was then vortexed for 1 min and centrifuged for 5 min. The supernatant layers were directly injected into a Sciex API 3000 LC/MS/MS system. 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 single-pot protein precipitation method is feasible for analyzing muraglitazar in human plasma. This method was subsequently used to analyze muraglitazar concentrations in human plasma samples from a first-in-man study. The method has also been validated in monkey and mouse plasma.

2. Experimental

2.1. Reagents and chemicals

Muraglitazar (Fig. 1) and its internal standard (a structural analogue) were provided by the Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute. The chemical structure of the internal standard was very close to muraglitazar; however, it cannot be shown for proprietary reasons. Acetonitrile (HPLC grade) and formic acid (88%, GR) were purchased from EM Science (Gibbstown, 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 plasma was purchased from Lampire Biological Laboratories (Pipersville, PA, USA).

2.2. Instrumentation

The robotic liquid handling system used was a Tecan Genesis RSP 150 Series with Gemini Software (Tecan US, Research Triangle Park, NC). The collection microtubes racked in 96-well format and the microtube caps were purchased from US Scientific (Ocala, FL).

The liquid chromatography separation system consisted of Shimadzu LC-10AD pumps (Columbia, MD, USA) and a Perkin Elmer Series 200 LC autosampler (Norwalk, CT, USA). The separation column was a Phenomenex C-18 (2) Luna (2 mm × 50 mm, 5 μm, Torrance, CA). Muraglitazar and its IS were separated isocratically, using a mixture of 20/80 (v/v) water

and acetonitrile containing 0.1% of formic acid, with flow rate of 0.3 mL/min. The injection volume was 5 μL and the run time was 2 min.

A Sciex API 3000 LC MS/MS system (Foster city, CA, USA) operating under Analyst 1.1 software was used. The electrospray ion source was run in a positive ionization mode for all experiments. The typical ion source parameters were: capillary 5.0 kV, declustering potential (DP) 36 V, focusing potential (FP) 150 V, entrance potential (EP) −10 V, collision energy (CE) 33 eV, collision cell exit potential (CXP) 14 V, deflector −350 V, channel electron multiplier (CEM) 2200 V, and source temperature 325 °C. Nebuliser gas (NEB), curtain gas (CUR) and collision gas (CAD) were set to 10, 12 and 4 of the state file parameters, respectively. Nitrogen gas was used for CUR, CAD, NEB, and auxiliary 2. The samples were analyzed via selected reaction monitoring (SRM). The monitoring ions were set as *m*/*z* 517–186 for muraglitazar and *m*/*z* 531–306 for its IS. The scan dwell time was set 0.15 s for both channels (Fig. 2).

2.3. Standard, QC and IS preparations

A 1 mg/mL standard stock solution was prepared by weighing the appropriate amount of muraglitazar and dissolving it into acetonitrile. A standard working stock solution of 10,000 ng/mL was prepared by appropriate dilution of the 1 mg/mL stock solution with drug-free human plasma. The final standard concentrations in human plasma were 1, 2, 12.5, 25, 50, 250, 500, 750, and 1000 ng/mL. Standard curves were prepared fresh daily.

A 1 mg/mL QC stock solution was prepared from a separate weighing and also dissolved in acetonitrile. Dilutions were used to prepare four levels of QCs in human plasma: 3, 400, 800, and 16,000 ng/mL (low, medium, high, and dilution QC). QCs were stored at −20 °C.

A 1 mg/mL stock solution of the IS was prepared in acetonitrile, and subsequently diluted with acetonitrile containing 0.1% formic acid to 50 ng/mL as the working internal standard solution.

2.4. Sample processing procedure

The Tecan was used to pipette an aliquot (0.1 mL) of each standard or QC to a rack containing 96 microtubes (1.2 mL). To each standard or QC sample, 0.3 mL of the working internal standard solution was added. The microtubes were capped with collection caps and vortexed for 1 min. The samples were then centrifuged for 5 min, and the supernatant layers were directly injected into the LC/MS/MS system.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample preparation

During method development, human plasma samples were initially precipitated with acetonitrile (1:3 in volume) containing the IS. The supernatant layer was aliquoted to a separate 96-well plate which was followed by dry-down, and reconstitution. The dry-down step is very time consuming and could also cause thermal labile compounds to degrade because of the excessive heat. Furthermore, it could lead to drug loss through adsorption and incomplete solubilization into the reconstitution solvents. To overcome those shortcomings, the sample transfer, dry-down and reconstitution steps were eliminated by direct injection of the supernatant layer into the LC/MS/MS system [19–23]. With proper adjustment of the autosample needle offset level, only the supernatants were injected without disturbing the precipitated proteins. The sensitivity of the direct injection (single pot) method was slightly better than that of the dry-down procedure even without addition of formic acid.

To optimize the clean-up procedure for the single-pot approach, different concentrations of formic acid (0.05–0.5%) were screened. It was found that a higher formic acid concentration generally provided cleaner samples, but a too high of concentration (at 0.5%) caused higher variability and inconsistency among standards and QC samples. By weighing in all aspects, 0.1% formic acid was selected for the method validation.

With the addition of 0.1% formic acid, protein aggregates were more gel-like (with a larger total volume) than solid pellets. Our observed gel-like appearance was consistent with what Filfil and Chalikian reported in acid-induced formational transitions of staphylococcal nuclease, a small globular protein [24], where they suggested that the protein adopted an expanded and considerably unfolded conformation. Under acidic conditions, the protein denature proceeded through a transient intermediate, and the whole process lasted for a few minutes. The presence of organic solvents, such as acetontirile used in our work, had a destabilizing effect to the intermediate [25]. Because of the transient intermediate, the exchange of the analyte and proteins continued between two phases (the supernatant layer and the gel) until equilibrium was reached. As the result of the transient intermediate, less amount of the analyte was trapped in the gel compared to direct formation of solid pellets. The single pot precipitation, using acetonitrile with 0.1% formic acid as precipitant, provided a slight better sensitivity compared to the method containing the dry-down and reconstitution steps, when the same dilution factor was used.

Since the supernatant layer and the protein gel coexisted throughout the sample analysis, a good understanding of the phase equilibrium time was critical for the success of the method. Therefore, the internal standard responses were carefully monitored throughout validation and sample analysis. The results showed that for an analytical run of 269 samples, the internal standard areas changed only 13%. This suggested that the equilibrium was reached in a few minutes, which was well before the first injection of any given run. Furthermore, muraglitazar and the IS were structurally very similar, therefore, the IS worked very well in tracking muraglitazar in the protein precipitation procedure.

With the improved sample clean-up procedure, the analytical column exhibited excellent stability. After more than 600 injections, there was only very slight increase of column backpressure, and no special treatment was required.

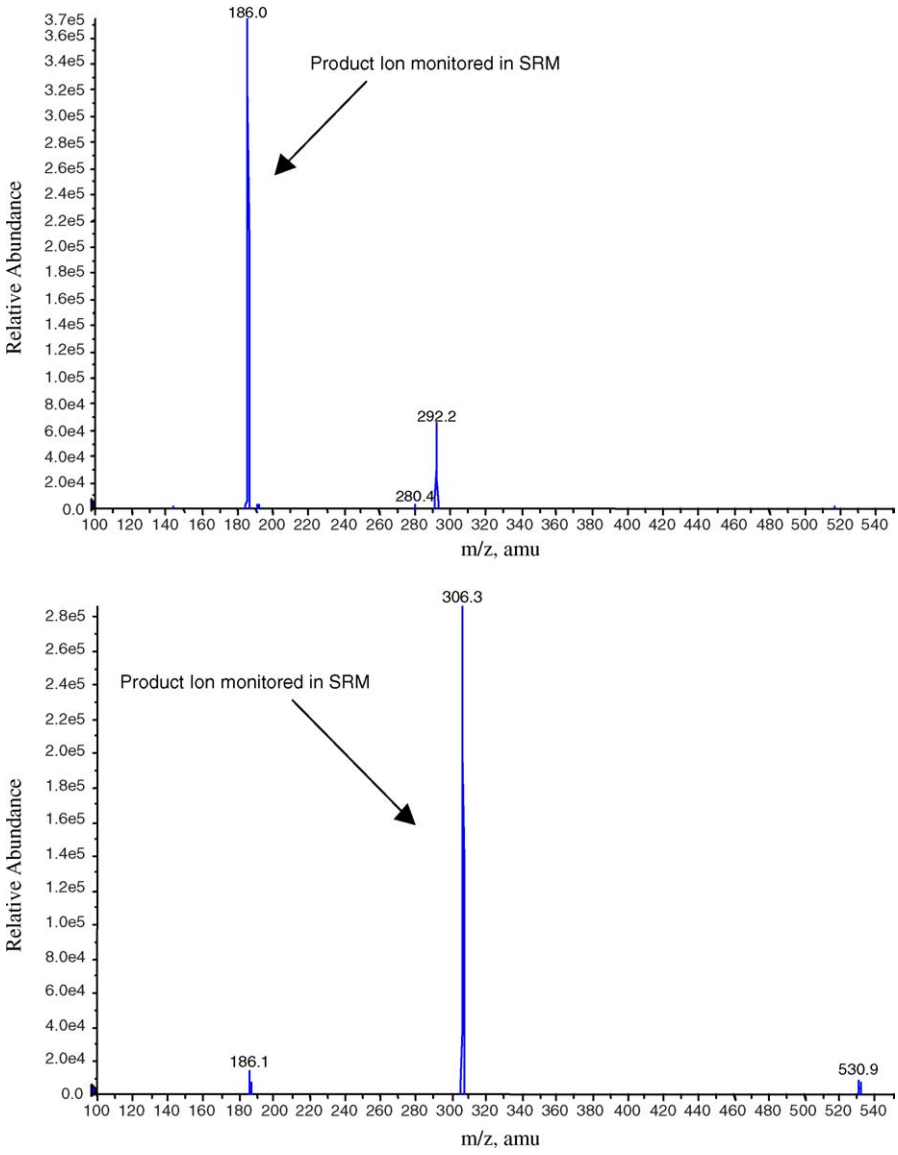

Fig. 2. Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for muraglitazar (top) and its internal standard (bottom).

3.1.2. LC conditions

3.1.2.1. Control of band broadening. With the current single-pot precipitation procedure, the processed samples contained 75% of acetonitrile, and the mobile phase used for this method contained 80% of acetonitrile. Under this condition, the method development results showed that for a 50 μL injection loop, 20 μL or less injection volume should cause no or minimal peak broadening. For the human plasma method, the injection volume was 5 μL. However, for the monkey and mouse plasma methods, the extracted samples with the single pot precipitation contained 95% of acetonitrile. With the same mobile phase, the method development results indicated that the injection volume had to be controlled under 12 μL to avoid any significant peak broadening. The actual injection volumes used were 4 and 10 μL for mouse and monkey plasma methods, respectively.

For a 50 μL injection loop, a 10 μL injection of a sample containing 95% acetonitrile corresponded to the addition of 9.5 μL acetonitrile. For this 10 μL injection, the loop contained 41.5 μL instead of 40 μL of acetonitrile in the loop if filled with the mobile phase. The relative increase of acetonitrile within the loop was about 3.75%. Such a small increment should not dramatically affect the peak width and shape. However, when a larger injection volume, such as 20 μL, was used, the peak showed significant broadening due to further increase of acetonitrile content in the loop. Under such a condition, proper integration of the peak areas became difficult. The similar observation was reported by Naidong et al. [26], where they experienced poor peak shape when stronger solvents were used in the sample preparation.

3.1.2.2. Detection sensitivity. The validated LLOQ for this method was 1 ng/mL. However, with the large signal to noise ratio of the LLOQ sample (Fig. 3C), it was predicted that the method could measure as low as 0.25 ng/mL samples without

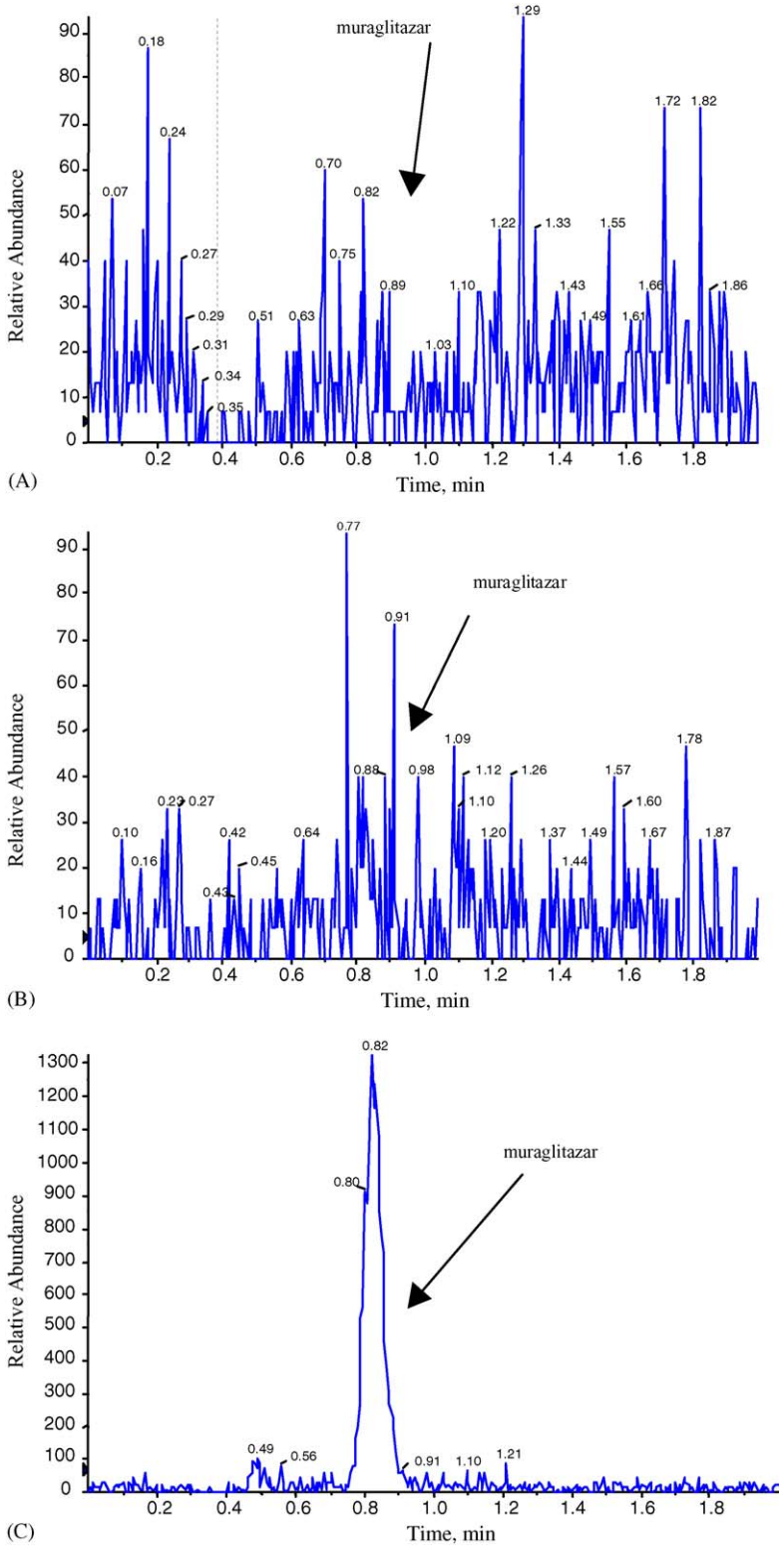

Fig. 3. Selected reaction monitoring chromatograms for muraglitazar obtained from: (A) blank human plasma; (B) human plasma containing only internal standard at 150 ng/mL and (C) human plasma containing muraglitazar at lower limit of quantitation (1.00 ng/mL) and its internal standard.

changing the extraction conditions. The method sensitivity could be further improved by using gradient elution. A gradient elution would re-focus the sample plug in the column compared with isocratic elution.

3.1.3. *Electrospray ionization tandem mass spectrometry*

Electrospray positive MS spectra for both compounds were dominated by the $[M + H]^+$ ions: *m/z* 517 for muraglitazar, and *m/z* 531 for IS. The MS/MS product ion spectra of the $[M + H]^+$

for muraglitazar and IS produced major product ions at *m*/*z* 186 and 306, respectively. Fig. 2 illustrates the fragmentation of each compound. Thus, the SRM used ranged from *m*/*z* 517 to 186 for muraglitazar, and from *m*/*z* 531 to 306 for IS. Since muraglitazar and the IS were eluted at approximately the same retention times (0.82 min for muraglitazar and 0.86 min for the IS), any fluctuations of ion source parameters encountered during the sample analysis were compensated.

3.2. Method validation

3.2.1. Standard curves

After the single-pot protein precipitation and the LC/MS/MS conditions were defined, a full validation was performed to assess the performance of the method. A nine-point calibration standard curve ranging from 1 to 1000 ng/mL of muraglitazar in human plasma was used in duplicate in each analytical run. Peak area ratios of muraglitazar to IS were used for regression analysis. A weighted ($1/x$) quadratic regression model, where x is the concentration of muraglitazar, was fitted to each standard curve. 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. The back-calculated concentrations of the 72 standards in the four analytical runs deviated no more than 14.0% from spiked concentrations except one rejected lowest standard due to an extraction error. In fact, more than 91% of standards were within 10.0% of the spiked concentrations. The regression coefficients (R-squared) for the four runs were greater than 0.997. 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 muraglitazar in human plasma.

3.2.2. Accuracy and precision

The accuracy and precision of the method was assessed by analyzing the low, medium and high QC samples (3, 400 and 800 ng/mL). A dilution QC sample (16,000 ng/mL), with a concentration higher than the upper limit of the standard curve range, was also analyzed. This QC sample was diluted 1:19 with control human plasma prior to processing and analysis. Five replicate samples at each concentration were analyzed in three separate runs, and triplicates were used for the fourth run. Accuracy was determined by calculating the deviations of the predicted concentrations from their spiked values. The intra- and inter-day precision was expressed as percent coefficient of variation (% CV).

Table 2 shows the summary of the individual QC data obtained in the four runs used for the validation. The deviations of the predicted concentrations from their spiked values were within ±11.0% for all of 72 QC samples and within 9.0% for 70 out of 72 QC samples. To further assess accuracy and precision, a one-way analysis of variance (ANOVA) was performed for the first three runs, and the results are shown in Table 3 (top panel). The intra-day precision was within 3.5% CV, and the inter-day precision was within 4.1% CV. The assay accuracy was within ±3.3% of the spiked values. Since QC samples are a good representation of study samples, similar precision and accuracy are expected from study samples. The QC data indicated that the single-pot protein precipitation, LC/MS/MS method was accurate and precise in the determination of muraglitazar concentrations in human plasma.

3.2.3. Lower limit of quantitation

To establish the LLOQ, six different lots of control human plasma were spiked at 1 ng/mL to obtain six LLOQ samples. The LLOQ samples were processed and analyzed with a stan-

Table 1
Individual standard curve concentration data for muraglitazar in human plasma

Spiked concentration	Run 1		Run 2		Run 3		Run 4	
	Predicted concentration	%Dev	Predicted concentration	%Dev	Predicted concentration	%Dev	Predicted concentration	%Dev
1	0.94	−6.0	0.97	−3.0	1.02	2.0	1.43	[a]
1	1.04	4.0	0.94	−6.0	0.86	−14.0	1.01	1.0
2	2.01	0.5	1.94	−3.0	1.98	−1.0	2.18	9.0
2	1.80	−10.0	1.97	−1.5	1.88	−6.0	1.91	−4.5
12.5	12.9	3.2	12.7	1.4	13.4	7.2	13.2	5.9
12.5	13.4	7.2	13.4	6.9	13.0	4.1	11.0	−11.9
25.0	25.7	2.7	24.8	−0.9	28.1	12.3	27.9	11.4
25.0	24.9	−0.3	26.1	4.4	24.1	−3.6	23.0	−7.9
50.0	49.7	−0.6	48.8	−2.5	51.9	3.7	51.5	3.1
50.0	49.1	−1.8	51.0	1.9	48.3	−3.4	46.9	−6.2
250	260	4.1	249	−0.3	257	2.7	263	5.2
250	248	−0.8	265	6.0	247	−1.4	232	−7.4
500	484	−3.1	474	−5.3	500	−0.1	542	8.5
500	491	−1.8	506	1.2	479	−4.1	474	−5.3
750	769	2.5	711	−5.2	777	3.6	776	3.5
750	753	0.3	780	4.1	741	−1.2	715	−4.7
1000	1023	2.3	963	−3.7	1008	0.8	1039	3.9
1000	971	−2.9	1051	5.1	988	−1.2	961	−3.9

Concentration: ng/mL.
[a] Sample deactivated–extraction error.

Table 2
Individual quality control sample concentration data for muraglitazar in human plasma

Spiked concentration	Run 1		Run 2		Run 3		Run 4	
	Predicted concentration	%Dev	Predicted concentration	%Dev	Predicted concentration	%Dev	Predicted concentration	%Dev
3	3.26	8.7	3.09	3.0	2.93	−2.3	3.11	3.7
3	3.09	3.0	3.13	4.3	3.03	1.0	2.67	−11.0
3	3.31	10.3	3.07	2.3	2.92	−2.7	2.87	−4.3
3	3.24	8.0	3.06	2.0	2.98	−0.7		
3	3.18	6.0	3.24	8.0	2.93	−2.3		
400	419	4.7	386	−3.6	392	−2.0	380	−5.1
400	421	5.1	387	−3.3	402	0.5	408	1.9
400	405	1.3	383	−4.3	396	−1.1	381	−4.9
400	400	0.1	399	−0.4	395	−1.2		
400	422	5.5	392	−1.9	395	−1.3		
800	852	6.5	736	−8.0	807	0.9	764	−4.5
800	818	2.3	728	−9.0	775	−3.1	748	−6.5
800	806	0.7	757	−5.3	753	−5.9	730	−8.7
800	838	4.8	808	1.0	788	−1.5		
800	817	2.2	812	1.4	774	−3.3		
16000	16956	6.0	15262	−4.6	16544	3.4	16953	6.0
16000	16350	2.2	157745	−1.4	16425	2.7	16704	4.4
16000	16818	5.1	16748	4.7	16507	3.2	16425	2.7
16000	16884	5.5	16775	4.8	16526	3.3		
16000	16969	6.1	16743	4.6	16487	3.0		

Concentration: ng/mL.

dard curve and QC samples, and their predicted concentrations determined. The deviations of the predicted concentrations for all six LLOQ samples were within ±16.0% of the spiked value. A typical SRM chromatogram at the LLOQ is shown in Fig. 3C.

3.2.4. Specificity, matrix effect and recovery

Six different lots of control human plasma were analyzed with and without the IS to determine whether any endogenous plasma 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 plasma were found at the retention time or in the ion channel of either the analyte or the IS. Figs. 3A, B and 4 illustrate chromatograms of blank plasma.

Muraglitazar has a carboxylic acid functional group, so acyl glucuronide could be a major circulating metabolite. Acyl glucuronide and muraglitazar need to be chromatographically resolved, because acyl glucuronide could contribute to the muraglitazar response by converting back to the parent in the ion source. A sample containing acyl glucuronide was injected into LC/MS/MS, and the chromatogram obtained demonstrated that acyl glucuronide was well separated from the parent peak with the retention time of 0.65 min. Human ADME study revealed no significant circuiting acyl glucuronide metabolite in human plasma. Therefore, no acidification was used for human plasma sample collections.

Matrix effect was assessed by comparing the average peak areas of six replicates of the neat solution with these of the lowest

Table 3
Accuracy and precision for muraglitazar in human plasma from the method validation (top) and during study sample analysis (bottom)

Spiked quality control concentration (ng/mL)	3	400	800	16000
Mean observed concentration	3.10	399.51	791.23	16517.95
%Dev	3.3	−0.1	−1.1	3.2
Inter-day precision (% CV)	4.1	3.0	3.6	1.1
Intra-day precision (% CV)	2.3	1.8	3.5	2.6
Total variation (% CV)	4.7	3.5	5.0	2.8
N	15	15	15	15
Spiked quality control concentration (ng/mL)	3	400	800	16000
Mean observed concentration	2.71	367.25	726.42	16433.97
%Dev	−9.7	−8.2	−9.2	2.7
Inter-day run precision (% CV)	0.0	3.5	4.0	0.0
Intra-day precision (% CV)	5.2	2.6	2.5	4.4
Total variation (% CV)	5.0	4.4	4.7	4.4
N	15	15	15	12

standard in plasma. The average peak areas of the lowest standard versus that obtained from the corresponding neat solution were 0.94 for the analyte and 0.86 for the IS, which indicated that there was approximately 6–14% matrix suppression for this method. Combined with the fact that there was no significant lot-to-lot variation in LLOQ and specificity results, it was concluded that such a low matrix effect did not compromise the performance of the method [8,26,27].

The recovery of the analyte from human plasma was determined at 3 and 800 ng/mL by comparing the response ratios of human plasma spiked with the analyte prior to extraction with those spiked post-extraction. The recoveries at 3 and 800 ng/mL were 86 and 95%, respectively.

3.2.5. Stability

The stability data were also generated to assess muraglitazar stability under its storage/processing conditions. Plasma samples containing two levels of muraglitazar were used for the stability experiments. In human plasma, muraglazar was found to be stable for at least 1.5 years at −20 °C, for at least 72 h at room temperature, as well as during three freeze-thaw cycles. The processed samples were also stable for at least 72 h at room temperature.

3.3. Applications

This method has been successfully applied for the determination of muraglitazar plasma concentrations for samples obtained from a first-in-man study. More than 600 plasma samples were analyzed within five separate runs, and all of them passed the batch acceptance criteria. A one-way ANOVA analysis was performed for the five runs, and the results are shown in Table 3 (bottom panel). The intra-day precision was within 5.2% CV, and the inter-day precision was within 4.0% CV. The assay accuracy was within ±9.7% of the spiked values. A representative chromatogram of a study sample is shown in Fig. 5. These numbers

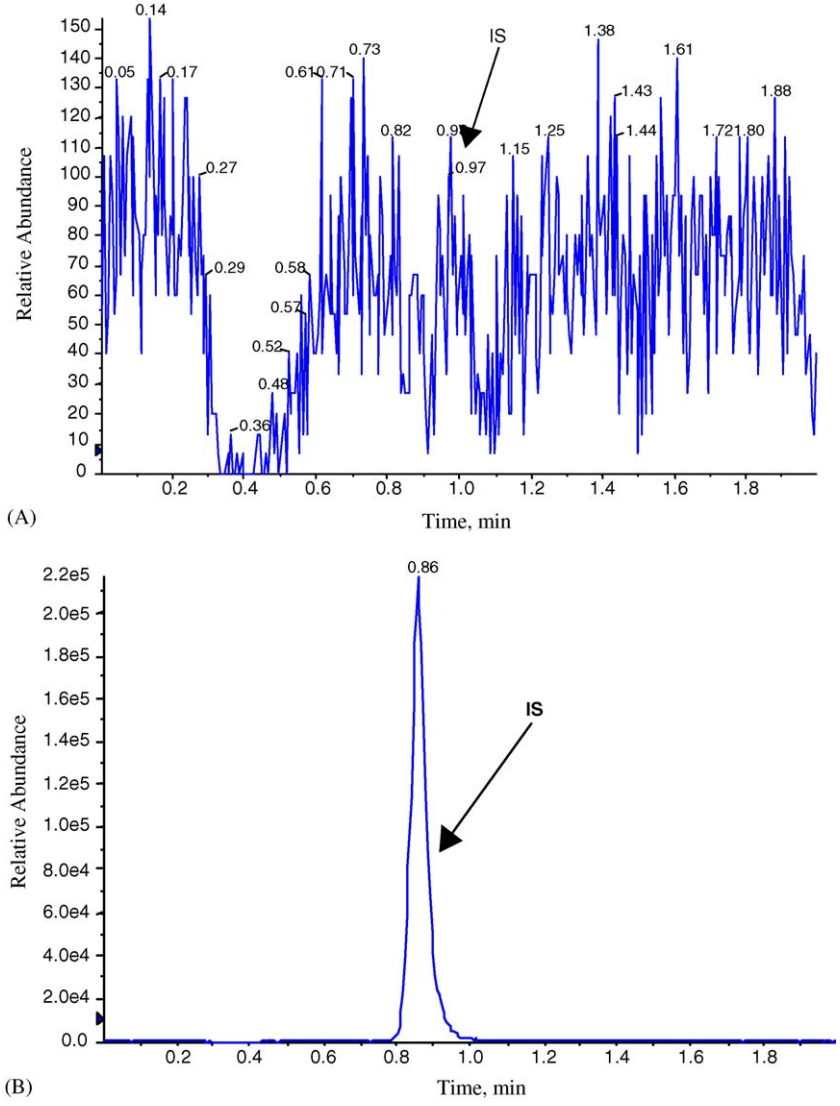

Fig. 4. Selected reaction monitoring chromatograms for the internal standard of muraglitazar obtained from: (A) blank human plasma and (B) human plasma containing only the internal standard.

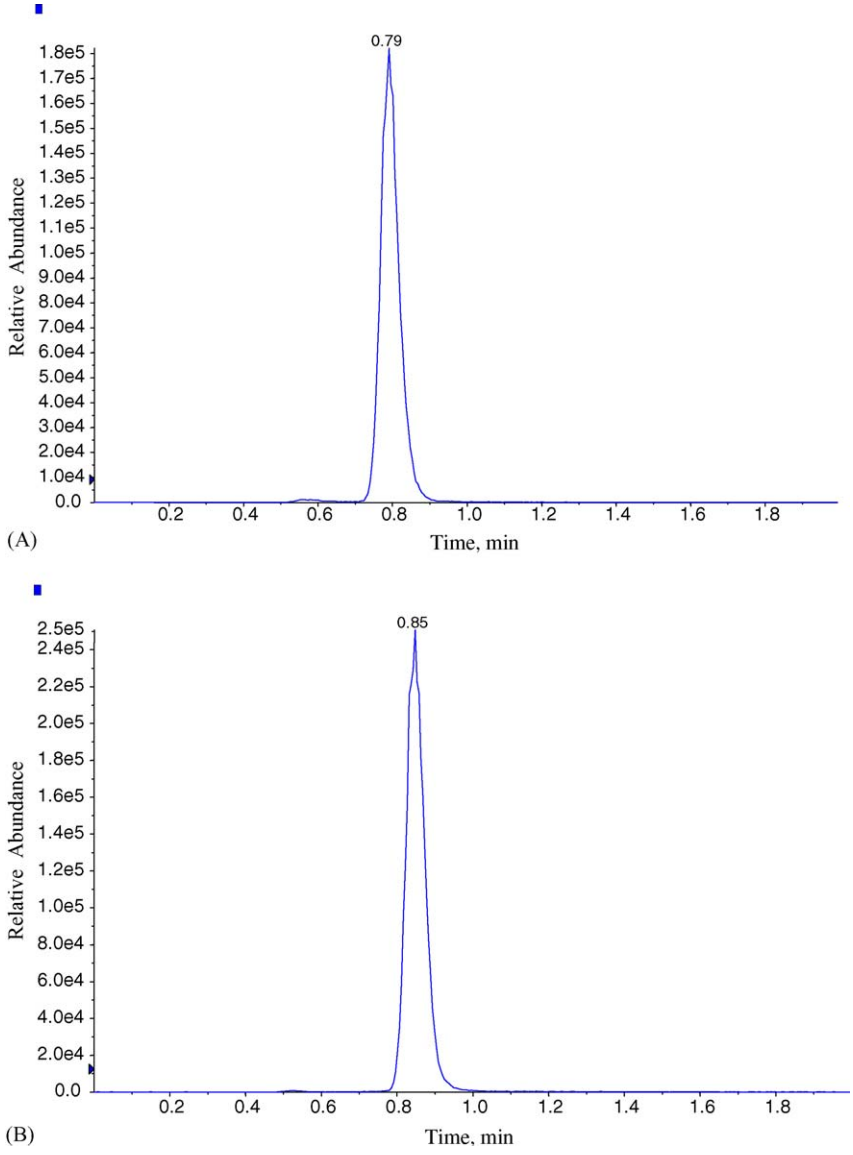

Fig. 5. A representative chromatogram of a study sample from first-in-man study: (A) analyte channel and (B) the IS channel.

Table 4
Accuracy and precision data for muraglitazar in monkey plasma (top) and mouse plasma (bottom) validations

Spiked quality control concentration (ng/mL)	3	400	800	16000
Mean observed concentration	3.05	388.41	779.15	15567.23
%Dev	1.7	−2.9	−2.6	−2.7
Between run precision (% CV)	3.9	5.7	5.0	4.6
Within run precision (% CV)	8.2	5.0	3.6	2.3
Total variation (% CV)	9.1	7.6	6.2	5.2
N	18	18	18	15
Spiked quality control concentration (ng/mL)	15	2000	4000	40000
Mean observed concentration	15.72	1982.42	4106.57	40501.68
%Dev	4.8	−0.9	2.7	1.3
Between run precision (% CV)	5.6	10.3	4.9	5.0
Within run precision (% CV)	2.9	1.8	3.2	3.8
Total variation (% CV)	6.3	10.5	5.9	6.3
N	21	21	21	15

are comparable to that obtained during the method validation (refer to Section 3.2.2). As described in Section 3.1.2, this single-pot protein precipitation, LC/MS/MS method has been validated in monkey and mouse plasma for the determination of muraglitazar. Monkey and mouse plasma validation data are shown in Table 4 (top and bottom panels). For monkey plasma method, the intra-day precision was within 8.2% CV, and the inter-day precision was within 5.7% CV. The assay accuracy was within ±2.9% of the spiked values. For mouse plasma method, the intra-day precision was within 3.8% CV, and the inter-day precision was within 10.3% CV. The assay accuracy was within ±4.8% of the spiked values. No significant differences in method performance were observed across these species (human, monkey, and mouse) tested.

4. Conclusions

We have demonstrated a method for the determination of muraglitazar in human plasma using a 96-well single-pot protein precipitation method and a rapid LC/MS/MS analysis. This method effectively eliminated three time-consuming steps needed for a traditional protein precipitation method: sample transfer, dry-down, and reconstitution before the injection, yet still preserved all the benefits of the traditional protein precipitation: simplicity and high throughput. The method possessed excellent precision and accuracy, and was proved to be rugged and reliable. The actual sample analysis results further demonstrated that this approach was well suited for real life applications. This single-pot protein precipitation, LC/MS/MS method has also been validated in monkey and mouse plasma for the determination of muraglitazar.

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