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Determination of chiglitazar, a dual alpha/gamma peroxisome proliferator-activated receptor (PPAR) agonist, in human plasma by liquid chromatography-tandem mass spectrometry

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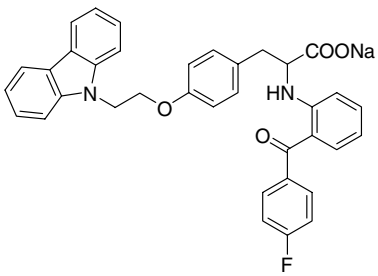
Chiglitazar is a dual alpha/gamma peroxisome proliferator-activated receptor (PPAR) agonist. A LC-MS/MS method for the determination of chiglitazar was developed and validated. The assay used 0.2 mL of plasma. 90% acetonitrile containing internal standard was used for protein precipitation. The mobile phase contained 70/30 (v/v) of methanol and water at a flow rate of 0.25 mL/min. Detection was by negative ion electrospray tandem mass spectrometry on a Sciex API 3000. The standard curve, which ranged from 2 to 1500 ng/mL, was fitted to a 1/x weighted quadratic regression model. The validation results demonstrated that the method was sensitive, rapid, selective and robust and provided satisfactory precision and accuracy. The method has been successfully used for the analysis of clinical samples in pharmacokinetic studies of chiglitazar.

1. Introduction

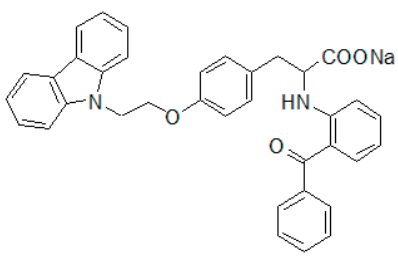
Chiglitazar is a novel dual alpha/gamma peroxisome proliferator-activated receptor (PPAR) agonist that been designed and composed by Shengzhen Chipscreen Biosciences Ltd. (China) and gained patent (Liao et al. 2003; Lan et al. 2004). By activating both PPAR-alpha and PPAR-gamma receptors, chiglitazar simultaneously lowers plasma glucose, improves insulin sensitivity and corrects dyslipidaemia (Lu et al. 2003). Chiglitazar is as a new alternative to existing treatments for type 2 diabetes and associated lipid abnormalities. Because chiglitazar is a new drug, no method of analysis has been reported for chiglitazar determination in human plasma so far. Thus, a sensitive and reliable analytical method was needed to support its clinical program.

Some dual alpha/gamma PPAR agonists have been analyzed in some different ways. Yao et al. reported a method for the determination of muraglitazar in monkey plasma using protein precipitation and LC-MS (Yao and Srinivas 2006). Xue et al. developed two methods to analyze muraglitazar in human plasma samples. One method used a single-pot liquid-liquid extraction (LLE) with hydrophilic interaction liquid chromatography/tandem mass spectrometry (HILIC-MS/MS) (Xue et al. 2006a). The other method was to use protein precipitation with LC-MS/MS (Xue et al. 2006b). Ragaglitazar was analyzed in human samples using liquid-liquid extraction and HPLC-UV (Kota et al. 2002). HPLC-MS/MS with solid phase extraction was also used to analyze ragaglitazar (Andersen and Nielsen 2003). Tesaglitazar was determined in blood plasma with an analytical method using solid phase extraction and LC-MS (Svennberg et al. 2003). MK-0767 was analyzed in human plasma using solid phase extraction and LC-MS/MS (Song et al. 2004).

Combining powerful separation from HPLC with superior selectivity and sensitivity from a mass spectrometer, LC-MS/MS is one of the most useful techniquies to quantitate drugs and their metabolites in human or animals fluids. The aim of the present paper was to develop and validate a sensitive, rapid and robust LC-MS/MS method for chiglitazar determination in human plasma. The lower limit of quantitation (LLOQ) for this method was 2 ng/mL and



Chiglitazar



Internal Standard

linearity range was 2–1500 ng/mL. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained from clinical pharmacokinetic studies.

2. Investigations, results and discussion

2.1. Method optimization

2.1.1. Sample preparation

To optimize the method of protein precipitation, methanol, acetonitrile, and a mixture of acetonitrile and mobile phase (9 : 1, v/v) as solvents were screened. Using methanol or acetonitrile, we found that acetonitrile was the more suitable solvent to precipitate protein than methanol, but plasmas were subject to form package. Therefore, methanol and acetonitrile were not feasible for precipitating protein. Our experiments showed that a mixture of acetonitrile and mobile phase (9 : 1, v/v) was the most effective and suitable solvent to precipitate protein as no package was produced in human plasma. Meanwhile, chiglitazar and the internal standard (IS) were structurally very similar, therefore, the IS worked very well in tracking chiglitazar in the protein precipitation procedure.
With the method, the analytical column exhibited excellent stability. After more than 600 injections, there was only very slight increase of column back-pressure, and no special treatment was required.

2.1.2. Choice of internal standard

We examined three compounds for that we would select the most suitable one from them as an internal standard. The MS/MS system for rosiglitazone maleate used the positive electrospray ion mode. It was opposite to that of chiglitazar and unfit for mass spectrometer scan. The sensitivity of CS088, a compound provided by Shengzhen Chipscreen Biosciences Ltd., was very poor and the retention time was too long. Finally, a structural analogue of chiglitazar, also provided by Shengzhen Chipscreen Biosciences Ltd., was proved to be the most suitable compound as an IS.

2.1.3. LC conditions

In order to find suitable chromatographic conditions of the method, solutions of mobile phase were tested. A mixture of 35% of 0.2% formic acid and 65% of methanol was used as the mobile phase. The peak shape of chiglitazar was very poor and the peak width was wide. Then 0.2% formic acid was adjusted to 0.1% formic acid, the peak shape and width was not improved. When 35% of 0.2% formic acid was replaced by water, the peak was about 10 times higher than with the two former solutions and sensitivity was increased. Meanwhile, with the increase of the proportion of methanol, the retention times of the analytes were decreased. During the experiment, we also used acetic acid and ammonium acetate to optimize shape and width of the chiglitazar peak. The peak shape and width were not improved dramatically. Combined with the fact that pure system of methanol/water was suitable for MS/MS system, a mixture of 70/30 (v/v) methanol and water was eventually used as the mobile phase.

2.1.4. Electrospray ionization tandem mass spectrometry

Electrospray negative MS spectra for both compounds were dominated by the $[M-Na]^-$ ions: m/z 571.2 for chiglitazar and m/z 553.3 for IS. The MS/MS product ion spectra of the $[M-Na]^-$ for chiglitazar and IS produced major product ions at m/z 527.5 and 509.3, respectively. Fig. 1 illustrates the fragmentation of each compound. Thus, the SRM used ranged from m/z 571.2 to 527.5 for chiglitazar, and from m/z 553.3 to 509.3 for IS. Since chiglitazar and the IS were eluted at approximately the same retention times (2.62 min for chiglitazar and 2.57 min for the IS), any fluctuations of in source parameters encountered during the sample analysis were compensated.

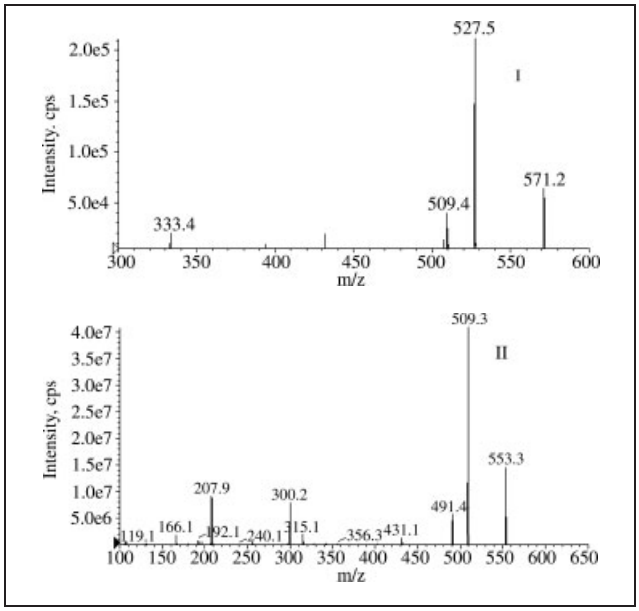


Fig. 1: Product scan spectra of chiglitzar (I) and internal standard (II)

2.2. Method validation

2.2.1. Standard curves

After protein precipitation method and 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 2 to 1500 ng/mL of chiglitazar in human plasma was used in duplicate in each analytical run. Peak area ratios of chiglitazar to IS were used for regression analysis. A weighted (1/x) quadratic regression model, where x is the concentration of chiglitazar, was fitted to each standard curve. The %CV at each level of chiglitazar varied from 0 to 14.00. The mean of the absolute value of percent deviation from the theoretical value of chiglitazar varied from 1.05 to 6.65. The %CV of the six slopes of chilgitazar was 9.29. The lowest coefficient of determination (γ) among the five calibration curves of chiglitazar was 0.9992 (mean = 0.9997). Thus, the calibration curves did not exhibit any non-linearity within the chosen range. 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 chiglitazar in human plasma.

2.2.2. Accuracy and precision

Inter- and intra-day accuracy and precision for assays were characterized by the three concentration levels of run in six replicates. Three levels were: low (2 ng/mL), medium (100 ng/mL), high (1500 ng/mL). Accuracy was assessed by calculating the percent deviation from the theoretical

Table: Intra- and inter-day accuracy and precision of chiglitazar in human plasma

Spiked quality control concentration	Low 2 ng/mL	Medium 100 ng/mL	High 1500 ng/mL
Intra-day accuracy and precision			
Mean	1.96	103.67	1598.33
SD	0.13	2.73	62.42
%CV	6.64	2.64	3.91
%Dev	−2	3.67	6.56
n	6	6	6
Inter-day accuracy and precision			
Mean	2.01	104.72	1621.11
SD	0.15	4.31	54
%CV	7.54	4.12	3.33
%Dev	0.5	4.72	8.07
n	6	6	6

concentration. Precision was determined by calculating the coefficient of variation for inter- and intra-day replicates.
The Table shows a summary of the assay results obtained in the three levels for the validation. As can be seen, the assay for chiglitazar was accurate and precise on an inter- and intra-day basis for each level. The greatest mean inter-day percent deviation was 14.00% for the low concentration. All concentration levels for chiglitazar had inter- and intra-day percent deviations less than 10% and the low concentration accuracy and precision results were within ± 10%.

2.2.3. Specificity, matrix effect and recovery

Six different lots of control human plasma were analyzed with and without standards to determine whether any endogenous plasma constituents interfered with the analyte or the IS. The degree of interference was assessed by inspection of the chromatograms. No significant interfering peaks from the plasma were found at the retention time or in the ion channel of the analyte or the IS (Fig. 2).
Matrix effect was assessed by comparing the average peak areas of six replicates of the neat solution with those of the lowest standard in plasma. The average areas of the lowest standard versus that obtained from the corresponding neat solution were 0.69 for chiglitazar and 0.71 for the IS, which indicated that there was approximately 29–31% matrix suppression for this method. Combined with the fact that there was no significant lot-to-lot variation and specificity results, it was concluded that such a low matrix effect did not compromise the performance of the method.
The recovery of the analyte from human plasma was determined at 100 and 1500 ng/mL by comparing the response ratios of human plasma spiked with the analyte

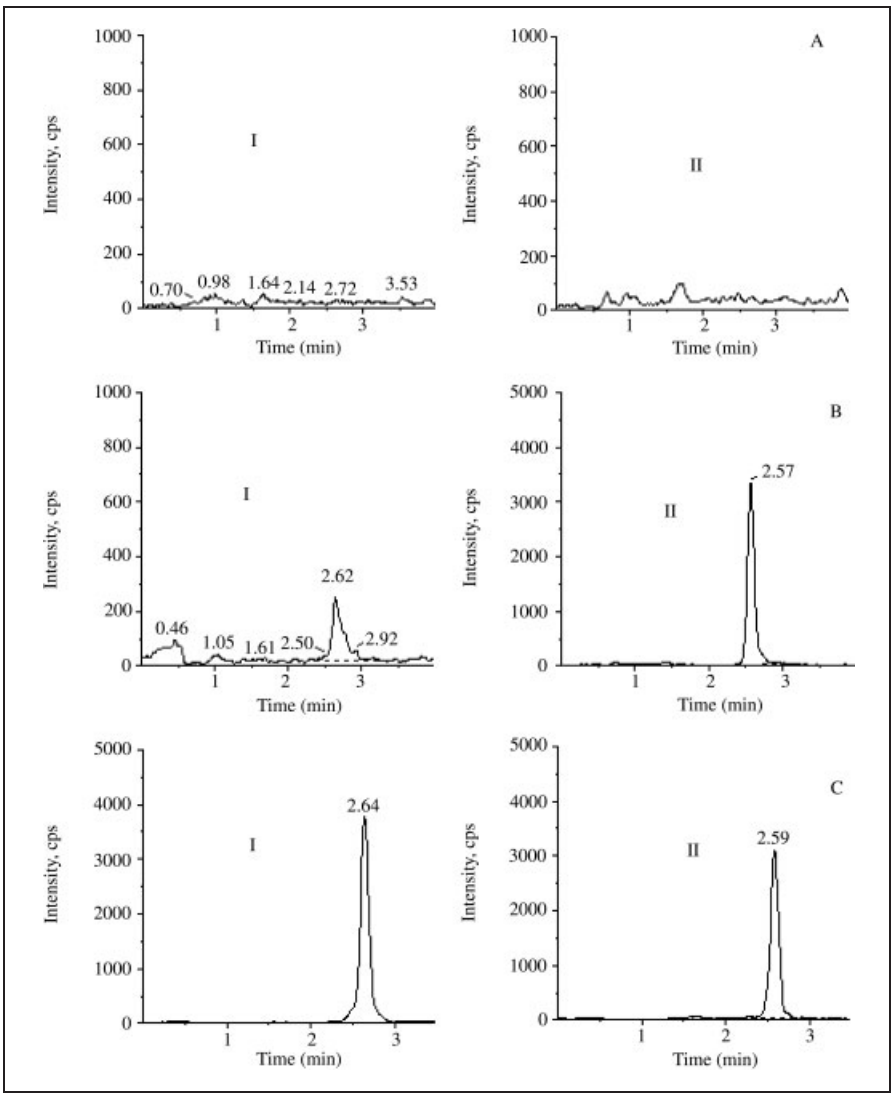


Fig. 2:
Chromatography of chiglitazar and internal standard (IS).
A: control blank plasma; B: human plasma standard at LLOQ (2ng/mL chiglitazar and 50ng/mL IS); C: plasma sample from a healthy subject after the administration of an oral single dose of 16 mg chiglitazar tablet
(I: chiglitzar; II: internal standard)

prior to extraction with those spiked post-extraction. The recoveries at 100 and 1500 ng/mL were 74 and 70%, respectively.

2.2.4. Stability

The stability of chiglitazar in human plasma was investigated using the low (2 ng/mL), medium (100 ng/mL) and high (1500 ng/mL) concentration levels. Test conditions included three freeze-thaw cycles and room temperature (4 h). Stability was also checked by extracting the appropriate concentration which had been maintained at specific temperature (4 °C) for the specified time and analyzing the extracts for chiglitazar concentration. A comparison was made with a control at the same level which had been stored at −20 °C and analyzed in the same analytical run. Additionally, extracted sample stability in the autosampler (4 °C) was tested by comparing the initial results with those determined after 12 h of autosampler storage. Deterioration of chiglitazar was defined as greater than a 15% difference of tested sample versus control at the sample nominal concentration.

There was no deterioration in chiglitazar at any concentration level (2, 100, 1500 ng/mL) for the various freeze-thaw cycles, suggesting that drug concentrations can be confidently determined in samples that had been thawed up to three times prior to analysis or that have been thawed and kept at ambient temperature for up to 4 h. The 4 h stability test at ambient temperature was performed since the plasma sample could stably stand on the bench for up to 4 h after thawing or before freezing.

The stability of chiglitazar in the extract was also tested after 12 h storage at 4 °C to allow for sample waiting or re-injection. Chiglitazar was considered stable to storage at 4 °C for 12 h if the relative error (RE) was less than 15% at all concentrations after the treatment. The results showed that chiglitazar was stable following storage at 4 °C for 12 h.

2.3. Clinical application

With approval of the Ethics Committee of Zhongshan Hospital, Fudan University, 12 healthy male Chinese volunteers participated in a three-period, rising, single-dose pharmacokinetic study. Before giving medicine, informed consent was obtained from all the volunteers. According to the study protocol, each of the volunteer received 8 mg, 16 mg, 32 mg chiglitazar tablet, respectively, at every period. The sample time was 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, and 48 h after giving tablets. The mean plasma concentration-time curves of 12 volunteers after the administration of an oral single dose of 8 mg, 16 mg and 32 mg chiglitazar tablet are shown in Fig. 3. The mean C_{max} were 165.42 ± 37.25, 339.92 ± 160.48 and 650.08 ± 190.56 ng/mL, respectively. The mean AUC_{0-t} were 1355.65 ± 555.16, 2597.66 ± 1143.81 and 5366.06 ± 1742.66 ng · h/mL, respectively. The mean t_{max} were 4.00 ± 0.95, 4.17 ± 1.03, 4.25 ± 0.97 h, respectively. The $t_{1/2}$ were 11.92 ± 4.32, 10.40 ± 3.02, 10.58 ± 2.24 h, respectively. These results showed that on the one hand mainly pharmacokinetic parameters (C_{max}, AUC_{0-t}) of chiglitazar have favorably linear relations to dose, on the other hand, t_{max} and $t_{1/2}$ had no relation to dose. As a result, chiglitazar shows linear pharmacokinetic characteristics in healthy Chinese volunteers.

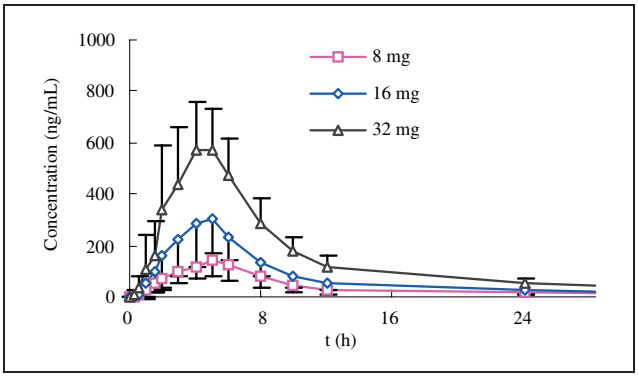


Fig. 3: Mean plasma concentration-time curves of 12 healthy volunteers after the administration of an oral single dose of 8 mg, 16 mg and 32 mg chiglitazar tablet

2.4. Conclusions

A LC-MS/MS method for the determination of chiglitazar in human plasma has been successfully developed and validated. By acetonitrile precipitation method, the procedure of sample preparation is easy and rapid and rugged. In addition, the 0.2 mL sample volume required for the method is reasonable. Finally, the analysis time for every sample is only 3.5 min. In conclusion, this method is sensitive, rapid, easy and robust. The method has been successfully applied to analyze thousands of clinical human plasma samples for pharmacokinetic studies of chiglitazar. Meanwhile, the method can also provide useful methodological reference for the same class drugs as muraglitazar, ragaglitazar, tesaglitazar and MK-0767.

3. Experimental

3.1. Reagents and materials

Chiglitazar (99.4% pure) and internal standard (IS, a structural analogue) (98.5% pure) were obtained from Chipscreen Biosciences Ltd. (Shengzhen, China). The chemical structure of IS is very close to chiglitazar and the difference between them is only one fluorine atom. Methanol and acetonitrile, both HPLC grade, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). In-house deionized water was prepared from tap water by our pharmaceutical department. Control human plasma used sodium citrate for anticoagulant was obtained from blood storage in our hospital.

3.2. Instrumentation

The liquid chromatography separation system consisted of LC-10Advp (pump), DGU-14AM (degasser) and SIL-HTc (autosampler) (Shimadzu Corporation, Japan). The separation column was a CAPCELL PAK C_8 MG (50 mm × 2.0 mm, 5 μm, Shiseido, Japan) with a security guarded column Octadecyl C_{18} (4.0 mm × 3.0 mm, Phenomenex, USA). Boifuge 28RS centrifuge was purchased from Heraeus Sepatech Co. (Osterode, Germany). An XW-80 votex was obtained from Shanghai Medical University Apparatus Co. (Shanghai, China). Chiglitazar and its IS were separated isocratically, using a mixture of 70/30 (v/v) methanol and water, with flow rate of 0.25 mL/min. The injection volume was 10 μL and the run time was 3.5 min.

A Sciex API 3000 LC/MS/MS system (Foster City, CA, USA) operating under Analyst 1.4 sofeware was used. The electrospray ion source was run in a negative ionization mode for all experiments. The typical ion source parameters were: declustering potential (DP) −100 V, focusing potential (FP) −350 V, entrance potential (EP) −12 V, collision cell exit potential (CXP) −12 V, collision energy (CE) −26 eV, and source temperature 450 °C. Nebulizer gas (NEB), curtain gas (CUR) and collision gas (CAD) were set to 9, 10 and 8 of the state file parameters, respectively. Nitrogen gas was used for NEB, CUR and CAD. The samples were analyzed via selected reaction monitoring (SRM). The monitoring ions were set as m/z 571.2 → 527.5 for chiglitazar and m/z 553.3 → 509.3 for its IS. The scan dwell time was set 0.2 s for both channels (Fig. 1).

3.3. Standard and IS preparations

A standard stock solution of chiglitazar (100 μg/mL) was prepared by weighing the appropriate amount of chiglitazar and dissolving it into

methanol. Working standard solutions of chiglitazar were prepared by combining aliquots of the standard stock solution and diluting with methanol. Human plasma calilbration standards of chiglitazar were prepared by spiking the working standard solutions into a pool of drug-free human plasma. The final standard concentrations in human plasma were 2, 5, 20, 50, 100, 200, 500, 1000, and 1500 ng/mL. Standard curves were prepared daily.

A stock solution of chiglitazar (100 μg/mL) was prepared from a separate weighing and also dissolving in methanol. Dilutions were used to prepare three concentration levels in human plasma: 2, 100, and 1500 ng/mL. The standards were prepared in plasma like the calibration standards and were stored at −20 °C.

A stock solution of the IS (100 μg/mL) was prepared in methanol, and subsequently diluted with acetonitrile containing 10% mobile phase to 50 ng/mL as the working internal standard solution.

3.4. Sample processing procedure

20 μL of each standard or sample was added into 1.5 mL labeled microcentrifuge tubes. To each standard or sample, 0.6 mL of the working internal standard solution was added. The tubes were capped and vortexed for 1 min. The samples were then centrifuged in Boifuge 28RS centrifuge at 18000 r/min and 4 °C for 10 min. Finally, the supernatant layers were filtered with micropore filter membrane (0.45 μm) and then transferred to labeled vials for injection and analysis.

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