Development and Validation of a Simple and Rapid HPLC Method for Determination of Pioglitazone in Human Plasma and its Application to a Pharmacokinetic Study

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Abstract

In this study, a new, simple, and reproducible high-performance liquid chromatographic method was developed for the determination of pioglitazone in human plasma. After liquid–liquid extraction with diethylether, samples were quantitated on a Nova-Pak C8 column using a mixture of acetonitrile–140mM K_2HPO_4 (40:60, v/v, pH = 4.45) as mobile phase with UV detection at 269 nm. The flow rate was set at 1.4 mL/min. Ethylparaben was used as internal standard and the total run time of analysis was approximately 7 min. The method was linear over the range of 25–1500 ng/mL of pioglitazone in plasma ($r^2 > 0.999$). The within- and between-day precision values were in the range of 2.4–6.8%. The limit of quantitation of the method was 25 ng/mL. The method was successfully used to study the pharmacokinetics of pioglitazone in healthy volunteers.

Introduction

Pioglitazone hydrochloride, (±)-5-{4-[2-(5-ethyl-2-pyridyl) ethoxy]benzyl}-2,4-thiazolidinedione hydrochloride salt (Figure 1), is an oral anti-hyperglycemic agent used in the treatment of type 2 diabetes (1, 2). Pioglitazone exerts its glucose-lowering effects by binding to peroxisome proliferator activated receptors gamma (PPARγ) and increasing the receptor sensitivity to insulin (3,4). Pioglitazone is rapidly absorbed and extensively metabolized by hydroxylation and oxidation to active and inactive metabolites in the liver (5).

Two high-performance liquid chromatography (HPLC) methods have been described for the determination of pioglitazone and its metabolites in human serum (6,7). The solid-phase extraction method was used in both methods and also the second method involves additional liquid extraction and gradiant HPLC, which is a time consuming process. The run time of these methods is more than 20 min. Another HPLC method was also reported for the determination of pioglitazone in human plasma by Sripalakit et al. (8) with a reasonable quantitation limit and run time using solid-phase extraction sample preparation method. Liquid chromatography with tandem mass spectroscopy (LC–MS–MS) was also reported for determination of pioglitazone with a low limit of quantitation (9,10).

Due to the expense and unavailability of LC–MS–MS in all laboratories, the purpose of the present study was to develop a simple and routine isocratic HPLC method with UV detection for determination of pioglitazone in human plasma after liquid–liquid extraction of drug. The developed method was used to study the pharmacokinetics of pioglitazone tablet in healthy male volunteers.

Experimental

Chemicals

Pioglitazone hydrochloride was from Biocon (Bangalore, India, Lot No: B-0620052/04401) and kindly provided from Osvah Pharmaceutical Company (Tehran, Iran). Ethylparaben (internal standard, I.S.), acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). All reagents and solvents used were analytical grade except acetonitrile and methanol, which were HPLC grade and used without any further purification. Distilled water was purified by a Millipore system MilliQ.

Instrumentation

The HPLC system consisted of a 515 pump, 710 plus Autosampler, and a variable 480 UV Detector all from Waters (Milford, MA). The data processing system was a multi-channel Chrom & Spec software for chromatography, version 1.5 x.

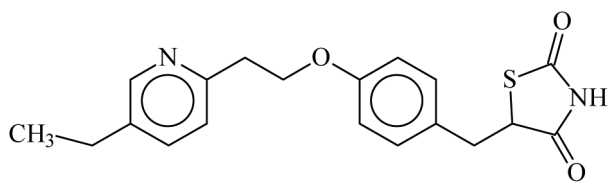

Figure 1. Chemical structure of pioglitazone.

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Chromatographic conditions

Chromatographic separation was achieved using a Nova-Pak C8, 4 µm column (250 mm × 4.6 mm, Waters). The isocratic mobile phase pumped at a flow rate of 1.4 mL/min consisted of acetonitrile and 140mM KH_2PO_4 (40:60, v/v) with final pH = 4.45. The mobile phase prepared daily and degassed by passing through a 0.45 µm filter and ultrasonication for 10 min. All separations were performed at room temperature. Detection was performed at 269 nm.

Standard solutions

Stock pioglitazone standard solution was prepared by dissolving appropriate amount of pioglitazone hydrochloride in 100 mL methanol to give a final concentration of 200 µg/mL. A series of pioglitazone standard solutions (0.25, 0.5, 1, 2, 5, 10, and 15 µg/mL) were prepared by subsequent dilution. A solution of internal standard (IS) was prepared by dissolving ethylparaben in methanol to a final concentration of 2 µg/mL. All these solutions were stored at 4°C.

Sample preparation

To a 250 µL of plasma sample in a test tube, 25 µL of of pioglitazone standard solutions and 25 µL of IS were added and vortex-mixed for 5 s. Then, 5 mL of diethylether was added. The test tubes were vortex-mixed for 45 s and centrifuged at 3500 rpm for 6 min. The organic layer was transferred to a clean test tube, and 100 µL of 0.04M NaOH was added. The test tubes were vortexed for 10 s and centrifuged at 3500 rpm for 3 min. The organic layer was discarded, and 60 µL of the aqueous layer was injected to HPLC system. For pharmacokinetic studies, the same procedure was used, and 50 µL of the aqueous layer was injected to HPLC system.

Validation

Six series of standard calibration solutions were prepared by spiking 25 µL of pioglitazone standard solutions and 25 µL of IS in 250 µL of blank human plasma to give final concentrations over the range of 25–1500 ng/mL. The sample preparation and HPLC analysis was performed as described earlier. Calibration curves were constructed by plotting the measured peak area ratios of pioglitazone to the IS versus concentrations of standard samples and statistical analysis was performed.

To establish the within-day and between-day accuracy and precision of the method, three replicates of standard plasma solutions at three different concentrations (25, 200, and 1500 ng/mL) were assayed on one day and six separate days.

Extraction yield

Aliquots of 25 µL of pioglitazone standard solutions (0.5, 5, 15 µg/mL) and 25 µL of I.S. solution were added to two sets of three test tubes. To one set, 250 µL plasma was added and extracted according to the sample preparation method. The other set was adjusted to the same volume by 0.04M NaOH. Sixty microliters of each solution was injected into the HPLC system. The peak area ratios of the extracted samples and unextracted samples were compared.

Stability

The stability of pioglitazone standard plasma samples at two different concentrations (100 and 1000 ng/mL) was checked for the freeze-thaw cycles within 3 days and also storing the plasma samples frozen for 2 months. The stability of samples prepared for analysis at room temperature was also checked for 24 h. The concentration of pioglitazone in plasma samples was determined and compared with freshly prepared samples.

Sample collection

To test the applicability of the analytical method to pharmacokinetic studies, the plasma concentration of pioglitazone was measured in human plasma after administration of an oral single dose of actos TM 45 mg (Takeda Europe R & D Center Ltd, UK, Batch Number: 1250003 S). Twelve healthy adult male volunteers (age: 28.3 ± 3.4 years, height: 173.5 ± 6.4 cm, body mass:

Table I. Statistical Data of Calibration Curves of Pioglitazone in Spiked Plasma (n = 6)

Parameters	Pioglitazone
Linearity	25–1500 ng/mL
Regression equation	$Y = 0.0034x - 0.069$
SD of slope	7.5×10^{-5}
RSD of slope (%)	2.22
SD of intercept	0.010
Correlation coefficient	0.9996

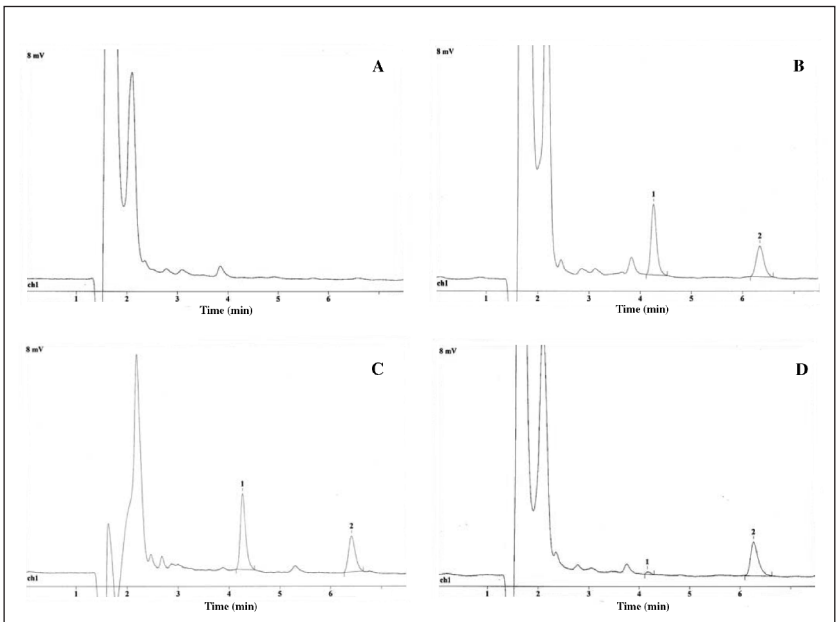

Figure 2. HPLC chromatogram of pioglitazone and IS drug free plasma (A); plasma sample spiked with pioglitazone (500 ng/mL) and I.S. (B); human plasma sample, 8 h after oral administration of 45 mg pioglitazone (C); plasma sample spiked with pioglitazone (10 ng/mL, LOD) and IS (D). Peaks: 1, pioglitazone; and 2, IS.

77.4 ± 4.4 kg) were selected and participated in the study based on acceptable physical examination, medical history, and clinical laboratory test results. All subjects gave written consent to their participation after having been informed verbally by the medical supervisor about the experimental procedures. After an overnight fast, each subject received a single 45-mg oral dose of pioglitazone. Blood samples (5 mL) were drawn into heparinized test tubes immediately before (0) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 24, and 36 h following drug administration. Blood samples were centrifuged at 2000 g for 10 min, and plasma samples were separated and stored at –20°C until analysis. Before analysis, the plasma samples were thawed at 18°C.

Results

Chromatographic condition

Different mobile phase systems and reversed phase columns were used for determination of pioglitazone. The best results were achieved using a Nova-Pak C8 and a mobile phase consisting of phosphate buffer and acetonitrile. Using a C8 column instead of a C18 columns created sharper peaks, which resulted in the increased sensitivity of the method. Several compounds were tested and ethylparaben was chosen as an internal standard with an appropriate retention time and consistent and reproducible recovery. Typical chromatograms obtained from blank and plasma samples spiked with pioglitazone and plasma sample from a volunteer 8 h after an oral single dose of 45 mg pioglitazone are presented in Figure 2. Under the chromatographic conditions described, the drug and IS were well resolved in plasma samples and eluted at 4.2 and 6.2 min, respectively. No interfering peaks of endogenous plasma components were found at the retention time of pioglitazone or internal standard in blank plasma.

Table II. Precision and Accuracy of Method for Determination of Pioglitazone in Spiked Plasma (*n* = 18; three sets for 6 days)

Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	CV (%)	Er (%)
Within-day (*n* = 3)			
25	26.63 ± 1.81	6.78	6.52
200	195.29 ± 6.64	3.40	–2.36
1500	1516.04 ± 44.45	2.93	1.07
Between-day (*n* = 18)			
25	25.50 ± 1.53	6.00	2.00
200	196.18 ± 4.60	2.34	–1.91
1500	1510.90 ± 35.95	2.38	0.73

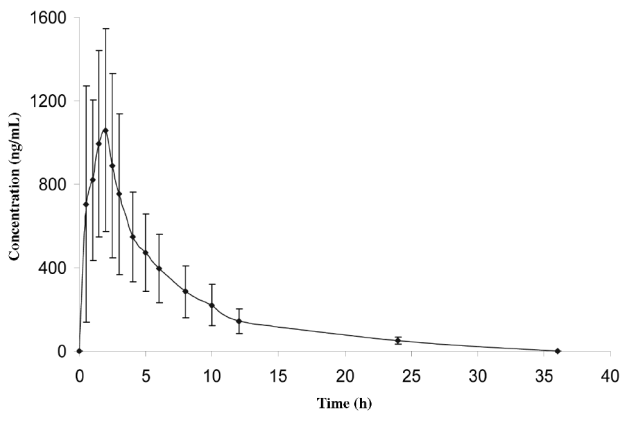

Figure 3. Mean ± SD of plasma concentration-time curve of pioglitazone following an oral single dose of 45 mg pioglitazone to 12 healthy volunteers.

Linearity

Calibration curves were constructed using six series of plasma samples spiked at concentration levels in the range of 25–1500 ng/mL. The linear relationship was obtained between the peak area ratio of pioglitazone to that of the internal standard versus the corresponding concentration, as shown by the equation presented in Table I. The linearity of the calibration curve is validated by the high value of the correlation coefficient.

Accuracy and precision

The accuracy and precision were determined by analyzing three samples of pioglitazone at 25, 200, and 1500 ng/mL in plasma on six separate days. Concentrations were determined using calibration standard curve prepared for pioglitazone in the range of 25–1500 ng/mL for each day. Within- and between-day data are given in Table II and indicate coefficient of variation (CV) values < 6.8% and Er < 6.5%.

Sensitivity

The limit of quantitation with CV < 6.8% was found to be 25 ng/mL for pioglitazone. The limit of detection that can be reliably detected with a S/N ratio of 3 was found to be 10 ng/mL.

Extraction recovery

Liquid–liquid extraction of pioglitazone from plasma was performed using diethylether as extracting solvent. The recovery of

Table III. Stability of Pioglitazone in Plasma Samples

Stability (*n* = 3)	Concentration (mean ± SD) (ng/mL) 100	1000
Freeze-thaw stability (3 cycles)		
Fresh sample	99.9 ± 1.7	1003.3 ± 11.3
Found	96.4 ± 1.7	981.5 ± 5.4
Deviation	–3.5	–2.2
Stability after 2 months		
Fresh sample	100.3 ± 2.6	1005.7 ± 10.9
Found	98.6 ± 1.0	991.9 ± 2.3
Deviation	–1.7	–1.4
Stability of analytical samples (24 h)		
Fresh sample	99.4 ± 1.7	1003.5 ± 10.9
Found	98.3 ± 1.5	996.2 ± 2.5
Deviation	–1.1	–0.7

pioglitazone and internal standard was determined. The mean recoveries of pioglitazone at concentrations of 50, 500, and 1500 ng/mL were 77.4 ± 3.3, 79.1 ± 1.9, and 83.2 ± 1.2%, respectively. Mean recoveries of internal standard were 91.8 ± 1.5 %.

Stability

Stability results of pioglitazone plasma samples and prepared analytical samples were presented in Table III. The deviation of the found concentrations with freshly prepared samples was lower than 4%. Freeze-thaw cycles had no significant effect on the concentration of drug.

Application to pharmacokinetic study

The average plasma concentration-time profile of pioglitazone after administration of a single 45-mg actos tablet to 12 volunteers was shown in Figure 3. The following pharmacokinetic parameters (mean ± SD) were provided for pioglitazone: C_{max} = 1150.92 ± 465.39 ng/mL, AUC_{0-36} = 6705.41 ± 2672.57 ng h/mL, T_{max} =1.67 ± 0.44 h, elimination half-life = 4.84 ± 1.94 h. The observed values of pharmacokinetic parameters were comparable to those reported in previous studies (8,9,11).

Discussion

Very few HPLC methods were reported for the determination of pioglitazone (6–8). Solid-phase extraction was used for sample preparation of these methods. In the first two methods, the metabolites were also determined and the run time was about 20 min. In the method reported by Sripalakit et al. (8), suitable run time (approximately 9 min) was achieved, but solid-phase extraction was used, and the limit of quantitation was 50 ng/mL in 1 mL plasma sample. Sensitive LC–MS methods were also reported for the determination of pioglitazone in plasma with lower quantitation limits, but the LC–MS instrument is not yet readily available in all laboratories.

The present method used isocratic reversed-phase HPLC with UV detection, which is the most convenient and common analytical method. A simple sample preparation procedure and a short chromatographic run time (approximately 7 min) along with low solvent consumption make this method suitable for processing multiple samples in a limited amount of time compared with the other reported methods. A lower quantitation limit (25 ng/mL using 250 µL of plasma sample) was also observed. The statistical evaluation of the proposed HPLC method revealed its good linearity and reproducibility and led us to the conclusion that it could be used for the rapid and reliable determination of pioglitazone in plasma in pharmacokinetic studies.

Conclusion

The present method is comparatively rapid, simple, reliable, and sensitive, allowing the processing of multiple determinations in a short time. Using UV detection with a low limit of quantification in low volume of samples makes this method very suitable for pharmacokinetic studies.

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Manuscript received September 30, 2007;
revision received December 25, 2007.