Development and validation of HPLC method for the determination of pioglitazone in human plasma

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Abstract: Pioglitazone is widely used for the management of type–II diabetes mellitus. The objective of the present study was to develop a simple and cost-effective HPLC method for the quantification of pioglitazone in human plasma. The mobile phase comprises of Acetonitrile, 0.1 M ammonium acetate and glacial acetic acid (25:25:1 v/v/v) at a flow rate of 1.2 mL/min., using Macherey-Nagel Column C_{18}, (dimensions: 5 µm; 250 × 4.6mm) with a guard column. The UV detector was set at 269nm. The method was validated according to FDA guidelines. The present method showed good linearity (R^2=0.9998) from 0.1 to 2.0µg/ml standards, with a limit of detection 0.1 µg/ml. Intra-day accuracy and precision in terms of %CV (range: 93.33% to 100.4% and 3.8% to 9.2%) and interday accuracy and precision (range: 94.1% to 102.7% and 4.8% to 9.6%) were in agreement with FDA guidelines. Freeze thaw stability showed that the plasma samples could be stored for one month at -20ºC without any appreciable degradation. The present method was successfully applied to the blood samples obtained from one volunteer after oral administration of 30 mg pioglitazone tablet. Some preliminary pharmacokinetic parameters were calculated. It is concluded that the present method could be conveniently used for the routine analysis of pioglitazone blood samples obtained in pharmacokinetics studies.

Keywords: Pioglitazone, RP-HPLC Method, Human Blood Plasma, Pharmacokinetic studies, Cost-effective

INTRODUCTION

Pioglitazone 5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl] methyl]-1,3-thiazolidine-2,4-dione is an anti-diabetic agent used for the management of type-2 diabetes-mellitus (Ghoreishia *et al.*, 2012). Comparatively to other anti-diabetics, pioglitazone has significantly lower risk of stroke and myocardial infarction which has been proved on the basis of the various clinical trials (Cheng *et al.*, 2017).

It has the ability to act as an agonist selectively for the nuclear receptor i.e., "peroxisome-proliferator-activated-receptor-gamma" (PPAR-γ) present in different tissues such as adipose tissue, liver, skeletal muscle, that are critical to insulin action (Macha *et al.*, 2015, Satheeshkumar *et al.*, 2014). It may also increase the receptor sensitivity to insulin (Souri *et al.*, 2008).

After oral administration, pioglitazone is rapidly absorbed (Brayfield, 2014) with a mean absolute bioavailability of 83% and maximum blood plasma concentration (C_{max}) reaches in around 1.5 h (Abushammala, 2015). Pioglitazone is highly bound to proteins (more than 99%). It is excreted in urine and faeces with a plasma half-life of about 7 hours (Brayfield, 2014).

Some methods are available for the quantification of pioglitazone in biological fluids which includes; HPLC methods (Souri *et al.*, 2008, Sripalakit *et al.*, 2006), liquid chromatography–electrospray ionization-tandem mass spectrometry method (LC–ESI-MS/MS) (Ni *et al.*, 2014) and liquid chromatography–tandem mass spectrometry methods (LC-MS/MS) (Sengupta *et al.*, 2017, Chinnalalaiah *et al.*, 2017, Abdel-Ghany *et al.*, 2017). HPLC methods reported involved either a tedious extraction procedure, which is time consuming or utilizes larger sample size that might ruin column life. Although LC-MS/MS and LC–ESI-MS/MS methods are highly sensitive and are able to detect nanogram levels in plasma, but costly and require sophisticated instruments that are usually not available in laboratories for routine analysis of plasma samples obtained in bioavailability and pharmacokinetic studies.

The aim of the present study was to develop or modify a simple, cost-effective, less time consuming method for the estimation of pioglitazone in blood plasma and to study pharmacokinetics of pioglitazone in one volunteer.

MATERIALS AND METHOD

Chemicals

Pioglitazone hydrochloride (Potency=99.80%) was kindly provided by Getz Pharma (Pvt.) Limited, Karachi, Pakistan. Ammonium acetate, Acetonitrile, Methanol, Glacial acetic acid of HPLC grade was obtained from Merck (Damstadt, Germany).

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Instrumentation

HPLC LC 10AT *VP* (Shimadzu, Kyoto, Japan), Communication Bus Module CBM 102 (Shimadzu, Kyoto, Japan) with software class GC 10 (Shimadzu, Kyoto, Japan), syringe micro-litre (Hamilton, Reno, Nevada, USA), balance (Sartorius, Goettingen, Germany), Swinney membrane filter (Millipore, Billerica, USA), Whirlimixer (Fisher Scientific, Loughborough, England), centrifuge machine (Heraeus, Osterode, Germany).

Chromatographic conditions

The Mobile phase was prepared by mixing Acetonitrile, 0.1 M ammonium acetate and glacial acetic acid in the ratio of (25:25:1 v/v/v), filtered through 0.45µ membrane filter and degassed in ultrasonic bath for 10-15mins. The column used was Macherey-Nagel Column C18, 5 µm (250 × 4.6 mm) with C18 guard-column (4.0 × 2.0 mm). The flow rate was 1.2 ml/min and the detector set at 269 nm.

Preparation of standard solutions

Pioglitazone standard stock solution was prepared in methanol (200µg/ml).The stock solution was diluted to prepare a working standard solution of 20µg/ml. Calibration curve samples were prepared serially from this stock solution in blood plasma to get six standard concentrations (0.1, 0.2, 0.3, 0.5, 1.0 and 2.0µg/ml). Three Quality Control (QC) samples independent of calibration standards; 0.15, 0.75 and 1.5µg/ml were also prepared in blank plasma from the same pioglitazone working standard solution as described above.

Plasma Protein precipitation for HPLC analysis

To 1ml plasma, 1ml acetonitrile was added, vortex for 2 minutes and centrifuged at 10,000 rpm for 15minutes. The supernatant liquid was separated, taken into another tube, filtered through 0.45µm filter and a 50µl sample was injected directly into HPLC column for analysis.

Method validation

The HPLC method was validated according to FDA guidelines (FDAGuidance, 2001; 2013).

System suitability

The system suitability was evaluated by injecting five consecutive injections of 0.4µg/ml drug plasma sample on each day of method validation (upto three days). The peak area, tailing factor and number of theoretical plates were determined to assess system suitability.

Specificity

Specificity of the method was estimated by analyzing six different independent samples of blank plasma. Interference of plasma components at the retention time of pioglitazone was observed.

Linearity

Linearity was determined by plotting standard calibration curve between peak area Vs plasma drug concentration in the range of 0.1 to 2.0µg/ml (0.1, 0.2, 0.3, 0.5, 1.0 and 2.0µg/ml). Two standard calibration curves were analyzed on 1st, 2nd and 3rd day of method validation which was evaluated by linear regression. Each calibration curve was back calculated to ascertain actual concentration.

In addition, lower end of the curve (0.1-1.0µg/ml) was also statistically analyzed to compare goodness of fit, slope and R^2 of pioglitazone in lower region Vs whole calibration curve.

Precision and accuracy

By replicate analysis of QC samples on 1st, 2nd and 3rd day, the intraday (within a day) and interday (between days) accuracy and precision was evaluated. Accuracy was calculated as the percentage of the calculated concentration compared to the true concentration. Precision were calculated and expressed as %RSD.

Absolute & relative recovery

Absolute recovery was obtained by comparing the peak area of spiked plasma QC samples with the peak area obtained from the equal strength of drug sample in mobile phase.

Relative recovery was determined by comparing the obtained concentrations of spiked plasma QC samples with that of actual concentration.

Sensitivity

Sensitivity of the method was measured by determining limit of quantification (LOQ) which is the lowest concentration that can be quantified with acceptable precision, accuracy and variability. Replicate drug plasma samples were injected at lower limit of quantification (0.1µg/ml).

Stability

Short term, long term, freeze and thaw drug stability was determined by placing spiked plasma samples at different concentration for specified period of time. For short term stability, QC samples were kept at room temperature (25 ± 2°C) for a time period expected for routine analysis (up to 12 hrs). Freeze and thaw drug stability was determined up to three freeze and thaw cycles. For freeze-thaw stability, fifteen QC samples of each concentration were stored at -20°C for 24hrs, five of them were thawed then analyzed, rest of them were refrozen and the same procedure was performed further two more time. For long-term stability of drug in plasma, samples were placed in refrigerator at −20°C for a period of 1 month.

Robustness & ruggedness

Parameters investigated were mobile phase ratio, flow rate, temperature and detection wavelength to determine robustness of the method (Mujtaba and Kohli, 2017). Similarly the method was analyzed by two different analysts in two different laboratories to determine ruggedness of the method (Dey *et al.*, 2017).

Subject and drug administration

A single dose of 30mg Pioglitazone tablet was given orally to one male volunteer after an overnight fast. The study was approved from the Ethical Review Committee of the Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan. A detailed amount of the Clinical Protocol will be described elsewhere.

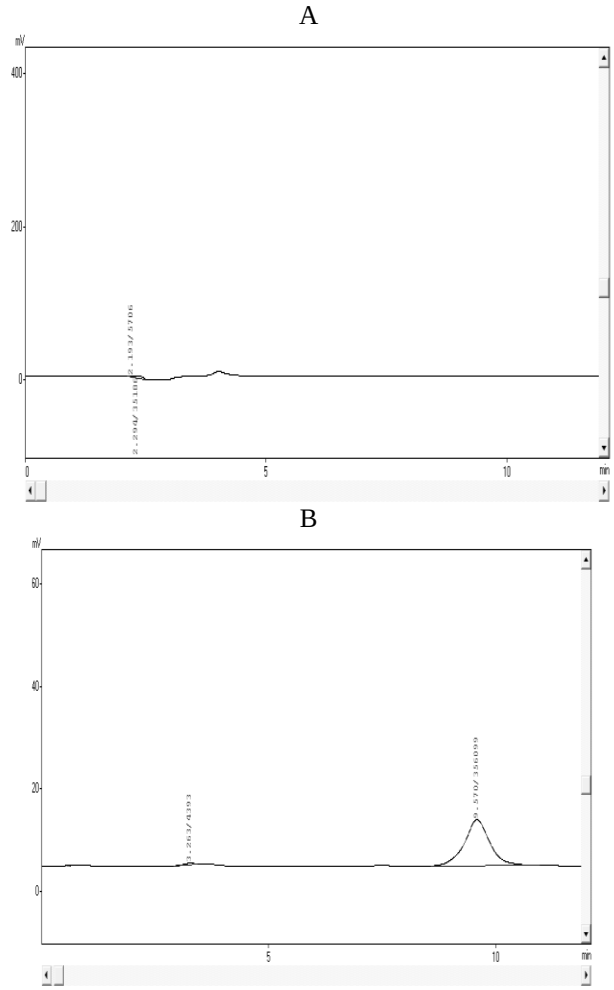

Fig. 1: Representative chromatogram of Blank Plasma from the volunteer (A) and plasma spiked with pioglitazone at 0.75 ug/mL (B)

Blood sampling and pharmacokinetic analysis

Approximately 10 mL blood was drawn according to the predefined protocol (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 12, 24, 36, 48 hrs). After centrifugation at 5000 rpm for 5 minutes, plasma was separated and stored at -20 °C for analysis. The drug was extracted by the method described above and analyzed using PK-Solver software (Zhang *et al*., 2010).

RESULTS

Peak retention time, peak area, number of theoretical plates and tailing factor of 0.4 µg/ml replicates were found within their specified limit which shows suitability of method for pioglitazone determination.

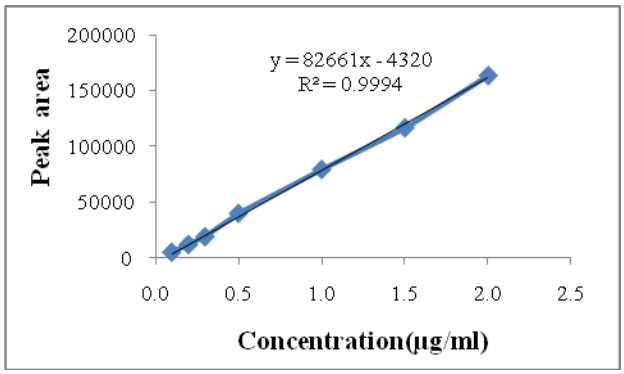

Fig. 2: Calibration curve of pioglitazone standards (whole curve)

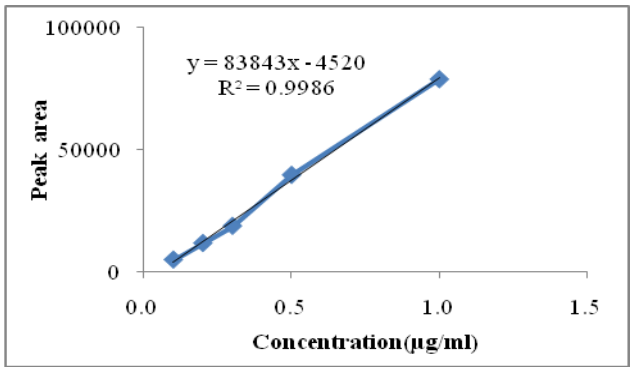

Fig. 3: Calibration curve of pioglitazone standards (lower end of the curve)

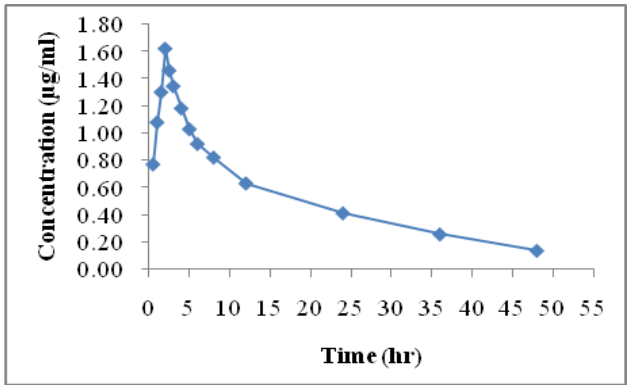

Fig. 4: Plasma Concentration time profile after oral administration of pioglitazone 30mg tablet to a healthy male volunteer.

Method Specificity was assessed by evaluating blank plasma from six different samples. Interference of any endogenous compound was not found at the retention time of the drug (fig. 1).

A linear relationship was observed between standards concentrations and peak area with average correlation coefficient > 0.99 for both the whole curve (fig. 2) and lower end of the curve (fig. 3) respectively. Back calculation of plasma calibration curve was found to be

Table 1: Intra-day accuracy and Precision of pioglitazone plasma samples

	Concentration(µg/ml)		
	0.15	0.75	1.50
Mean (n=5)	0.14	0.74	1.48
*SD	0.01	0.01	0.04
**CV (%)	7.02	1.10	2.91
Accuracy (%)	93.33	98.93	98.53

Table 2: Inter-day accuracy and Precision of pioglitazone plasma samples

	Concentration(µg/ml)		
	0.15	0.75	1.50
Mean (n=5)	0.14	0.74	1.52
*SD	0.008	0.01	0.07
**CV (%)	6.01	1.67	4.70
Accuracy (%)	92.44	99.20	101.29

Table 3: Absolute recovery of pioglitazone

Conc.	Peak Area of drug in plasma. Mean (n=5)	Peak Area of drug in Mobile Phase	Recovery (%)
0.15	8376.3	9569.7	87.53
0.75	59136	68441.8	86.40
1.50	139597.5	151338.7	92.24

n = 5

Table 4: Long term stability of pioglitazone in plasma at -20°C

	Concentration 0.15 µg/ml		Concentration 0.75 µg/ml		Concentration 1.50 µg/ml	
S No.	Initial Value	Conc. After1month	Initial Value	Conc. After1month	Initial Value	Conc. After1month
Mean(n=5)	0.14	0.13	0.732	0.73	1.488	1.484
*SD	0.01	0.01	0.016	0.015	0.024	0.021
**CV	7.14	4.09	2.24	2.02	1.37	1.20
Accuracy %	93.33	89.33	97.6	97.6	99.31	99.08
Stability %	-	95.71	-	100	-	99.77

*Standard deviation, **Coefficient of variance

92.62% to 109.84%. Both intraday and interday accuracy and precision was comparable (tables 1 and 2).

To assess absolute recovery, peak area of the spiked plasma sample was compared with that of drug sample in mobile phase. Three independent QC samples near to low, mid and high concentration used to calculate absolute recovery (table 3). Similarly relative recovery was determined to compare concentration of QC samples obtained with actual concentration which was found to be 92.0% to 98.63%.

For estimation of Sensitivity, the lowest concentration of calibration curve was analyzed (0.1µg/ml). It was found that drug was quantifiable in plasma at concentration of 0.1µg/ml with accuracy and precision of 93.60% and 5.37% respectively.

The developed HPLC method was analyzed on two HPLC's, present in different laboratories by two analysts. No significant difference was found between the results. This indicates that the method qualify ruggedness criteria. When deliberate changes were made to evaluate robustness of the method, results were found within the specified limit (NMT2% CV).

Short term stability was found to be 98.60% to 99.45%. Freeze-thaw stability at low concentration found to be 98.59% to 101.41%, at mid concentration; 99.46% to 100.54% and at high concentration 98.97% to 99.89% respectively. Long term stability was found to be ℘ 0.95% (table 4).

Some pharmacokinetic parameters derived from the plasma samples of one volunteer showed Cmax, Tmax, $T_{1/2}$ and AUC of 1.45µg/mL, 2 hrs, 18.2 hrs and 28.2µg/mL.hr respectively (fig. 4).

DISCUSSION

The objective of the present work was to develop a simple, cost effective HPLC method that could be conveniently used for the routine analysis of large number of plasma samples usually obtained in pharmacokinetic studies. Only few HPLC methods are available for the quantification of Pioglitazone in Plasma (Souri *et al.*, 2008, Sripalakit *et al.*, 2006).

Before starting the work, a thorough search of literature was carried out using various search engines to study methods available for the quantification of pioglitazone in biological samples. To the best of our knowledge, the HPLC methods reported to-date for pioglitazone are either difficult or requires larger samples after extraction. The liquid chromatography–tandem mass spectrometry (LC-MS/MS), liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) methods, although sensitive enough but are costly and not suitable for routine analysis.

USP has reported an *in-vitro* analytical method for the assay of pioglitazone in tablet dosage form (USP, 2014). In the present study, this method was selected and modified for the quantification of pioglitazone in human plasma. Initially the same mobile phase (Acetonitrile, 0.1 M ammonium acetate, glacial acetic (25:25:1 v/v/v) as reported in USP (USP, 2014) at a flow rate of 0.7 mL/min with UV detection at 269 nm was used to study the analyte behavior that resulted in a sharp peak with good symmetry. Thereafter, mobile phase composition was varied to get a optimize peak in plasma. Best resolution was found at the same composition as recommended by USP for *in-vitro* analysis of pioglitazone, therefore same mobile composition was selected for present study. Different extraction solvents such as perchloric acid, di-ethyl ether and acetonitrile were then tried in various compositions for the de-proteinization of Pioglitazone from plasma samples. Among all reagents tried, acetonitrile gave maximum recovery of Pioglitazone from plasma samples. Therefore it was chosen for the extraction of Pioglitazone form plasma.

Previously, mobile phases (HPLC) used for pioglitazone estimation comprises of either methanol, acetonitrile and mixed phosphate buffer (40:12:48, v/v/v), pH 2.6 at a flow rate of 1.2ml/min (Sripalakit *et al.,* 2006) or acetonitrile and potassium dihydrogen phosphate (40:60, v/v), pH 4.45 run at a flow rate of 1.4ml/min (Souri *et al.*,2008) and samples were analyzed at 269 nm. The mobile phase used here is simple to prepare and might not affect the column life for long time.

For the determination of specificity, six individual independent samples were analyzed separately to observe the interference of endogenous compounds at the retention time of pioglitazone. All runs showed absence of any interfering peak at the retention time of pioglitazone which was obtained around 9 minutes (fig. 1). This indicates that the method is specific for pioglitazone estimation without any interference to the endogenous components.

For linearity, six standards were run at the concentrations expected for the analyte in plasma samples. A close agreement of the observed and calculated values clearly indicates high index of correlation that resulted in almost same slope which shows the reproducibility of the current method. The whole curve alone Vs lower end of curve showed same goodness of fit that indicates no variability throughout the whole range selected for the present study. Both intra/interday accuracy and precision for the independent QC samples close to low, mid and high values of calibration curves revealed lower values in term of CV which is in line with FDA guidelines (FDA Guidance, 2001; 2013).

Likewise accuracy and precision, relative and absolute recovery values in mobile phase and plasma was also comparable. LOQ reported in previous HPLC methods are 25ng (Souri *et al.*, 2008) and 50 ng (Sripalakit *et al.*, 2006) respectively. The present work appears to be less sensitive comparatively to previous methods, yet sensitive enough to determine the plasma concentrations immediately after pioglitazone administration till the last sampling time.

The ruggedness of the method was investigated by independently running the method by two analysts. A close resemblance in peak area was found between the two laboratories which indicate that the method could be used in any laboratory and will not effect by different analysts or laboratory conditions. For robustness, HPLC method was slightly varied in terms of some common factor that might influence method performance. The data obtained revealed no variation in the performance parameters.

The pharmacokinetic parameters obtained are comparable with the previous studies (Wittayalertpanya *et al.,* 2006, Sripalakit *et al.,* 2006, Hiroi *et al.,* 2012). A detailed account of these parameters will be described elsewhere.

CONCLUSION

From the overall performance of the present HPLC method (development, validation and application), we might conclude that the proposed method could be successfully used for the routine analysis of blood samples. The method reported here is simple in terms of extraction and run time that would be economical and cost effective. It appears that still we need other methods that could quantify Pioglitazone concentrations in plasma

and other biological fluids with the required accuracy and precision as recommended by the regulatory authorities.

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