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Sensitive and Rapid HPLC Method for the Determination of Pioglitazone in Rat Serum

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ABSTRACT
A sensitive, accurate and rapid high-performance liquid chromatography with UV-visible detection (HPLC-UV) method for the determination of Pioglitazone in rat serum has been developed. Rosiglitazone was used as internal standard. Pioglitazone and Rosiglitazone are extracted from serum using a liquid–liquid extraction procedure using ethyl acetate. Isocratic separation of Pioglitazone and Rosiglitazone is carried out using a reversed-phase phenomenex C18 (250 mm × 4.6 mm, 5µm) column with mobile phase consisting of methanol and 30 mM ammonium acetate buffer (pH adjusted to 5 with ortho-phosphoric acid) in the ratio 60:40 (v/v) and quantified by UV detection at 269 nm. Analytical run time was less than 10 min. Mean recovery was 97.12% for 0.1-10µg/ml concentrations. The assay exhibited good linear relationship. Quantification limit was at 50ng/ml of Pioglitazone and accuracy and precision were over the concentration range of 0.1-10µg/ml. This method can be used for routine clinical monitoring of Pioglitazone.

Keywords: Ethyl acetate, HPLC, Pioglitazone, rat serum.

INTRODUCTION

Pioglitazone is an oral antidiabetic agent belonging to the class of thiazolidinediones that acts primarily by decreasing insulin resistance. It is a peroxisome proliferator-activated receptor-γ (PPARγ) agonist that increases transcription of insulin-responsive genes and thus increases insulin sensitivity. It is used in the management of type 2 diabetes mellitus. It improves sensitivity to insulin in muscle and adipose tissue and inhibits hepatic gluconeogenesis also improves glycemic control while reducing circulating insulin levels. Pioglitazone [(±)-5-[[4-[2-(5-ethyl-2- pyridinyl) ethoxy] phenyl] methyl]-2,4-] thiazolidinedione mono-hydrochloride belongs to a different chemical class and has a different pharmacological action than the sulfonylureas, metformin, or the α-glucosidase inhibitors. [1]

It is used both as monotherapy and in combination with sulfonylurea or insulin in the management of type 2 diabetes mellitus (non-insulin dependent diabetes mellitus, NIDDM). [2-4] Several liquid chromatography methods have been described in the literature to determine pioglitazone and its metabolites in biological fluids. The classical procedure used was high-performance liquid chromatography with ultraviolet detection (HPLC-UV) with run times of more than 20 min. [5-9]

Pioglitazone is well absorbed after oral administration at doses ranging from 15 to 45 mg, peak concentrations of Pioglitazone in the blood of healthy subjects are achieved approximately 1.5 h after oral drug administration. Pioglitazone is highly bound to serum proteins (approximately 97%), with a low tissue distribution and slow elimination (half-life approximately 9 h). Pioglitazone is extensively metabolized in the liver, with the majority excreted as inactive metabolites in the feaces. [10] Pioglitazone possesses a low molecular weight (MW; 392.90 Da) and a low aqueous solubility. The structure of Pioglitazone was shown in Fig. 1.

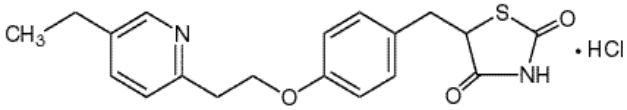

Fig. 1: Structure of Pioglitazone hydrochloride

The aim of the present study is to develop a simple, sensitive and rapid RP-HPLC method with UV detection for the quantitative determination of Pioglitazone in rat serum. The method uses HPLC-UV with Rosiglitazone as the internal

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standard. This method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy. This analytical method can be used for the estimation of Pioglitazone in biological samples.

MATERIALS AND METHODS

Chemicals and reagents

Pioglitazone and Rosiglitazone obtained from Dr. Reddy's laboratories, Hyderabad, India as gift samples. Methanol (HPLC grade), ammonium acetate and Ortho-phosphoric acid were purchased from Merck. Double distilled Water for analytical purpose was obtained from milli-Q R-O system. Serum samples were obtained from healthy rats

Preparation of standard solutions and calibration standards

Stock solution of Pioglitazone was prepared in methanol at a concentration of 1 mg/ml and was kept at -20°C. This stock solution was diluted with methanol to obtain the concentrations required for preparation of standard working solutions. Pioglitazone working solutions were in the range of 0.1µg/ml-10µg/ml. The internal standard was prepared by dissolving 50µg of Rosiglitazone in 1 ml of methanol. Samples for the determination of recovery, precision and accuracy were prepared by spiking quality control (QC) standard Pioglitazone concentrations (0.4, 0.8, 1, 4, 8µg/ml) and stored at -20°C.

Instrumentation

The HPLC system consisted of a Shimadzu LC- 10AT VP liquid chromatographic pump, Rheodyne injection port (Rheodyne, Cotati, CA,USA) with a 20 µl sample loop and SPD-10A VP UV-Visible spectrophotometer detector (Shimadzu, Kyoto, Japan). Data collection, integration and calibration were accomplished using Class VP chromatography Data system.

HPLC conditions

The chromatographic separation of Pioglitazone and internal standard (Rosiglitazone) were accomplished using 250x4.6mm phenomenex C18 5µm reverse phase analytical column. The mobile phase consisted of methanol and ammonium acetate (30 mM; pH 5) in the ratios of 60:40 v/v. Before use, the mobile phase was filtered by passing it through a 0.45µm filter and the filtrate is degassed by using bath sonicator. The mobile phase was pumped at an isocratic flow of 1 ml/min at room temperature. The peaks were determined using a UV detector set at a wavelength of 269 nm. All the procedures were performed at ambient temperature.

Extraction procedure

The serum samples were obtained from healthy rats. In 2ml micro centrifuge tube, 100µl of serum was added along with 100µl internal standard solution (Rosiglitazone 50µg/ml). The serum was precipitated by the addition of 200µl of ethyl acetate [11] and then tubes were vortexed for 2 min and centrifuged at 10,000g/min for 10min. The supernatant was transferred to a clean tube and the 20µl solution was injected into the HPLC.

Assay validation

The RP-HPLC assay validation was done as per ICH Q2A and Q2B guidelines. [12-13] These tests included determination of accuracy, precision, linearity, sensitivity and limit of detection, limit of quantification and recovery.

Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Standard calibration samples were prepared by making serial dilutions from the stock solution of Pioglitazone (1 mg/ml). Calibration curve of concentration versus peak area ratio was plotted at concentration range of 0.1µg/ml-10µg/ml.

The limit of detection was defined as the lowest concentration of Pioglitazone resulting in a peak height greater or equal to three times from background noise ($S/N \geq 3$). The LOQ was investigated in extracted samples from five different days. For the determination of LOQ, the percentage deviation and % RSD are to be less than 20%.

Precision and Accuracy

The precision and accuracy were determined by analyzing spiked standard and extracted samples at different concentrations ranging from 0.1µg/ml-10µg/ml. The precision of an HPLC method was determined as the coefficient of variation (%RSD) of intra- and inter-day. The intra-day precision was determined by analyzing the spiked standard and extracted samples prepared within a day. The inter-day precision was determined by analyzing the spiked standard and extracted samples analyzed on five different days. After concentrations were calculated by re-fitting peak area ratios obtained with different standard solutions into a derived regression equation from the set of these standard solutions, %RSD was determined at each concentration of the standard solutions from their average value and S.D. Accuracy (expressed as %RSD) was calculated as the percent difference between the amount of Pioglitazone added and found.

Recovery

The recoveries of Pioglitazone and Rosiglitazone in the extraction procedure were determined by comparing the peak areas obtained from an extracted sample spiked with known amounts of Pioglitazone and Rosiglitazone with those obtained from the pure compounds of the same concentrations in the solutions. The recoveries were determined in triplicate at five concentrations (0.4, 0.8, 1, 4, 8µg/ml).

RESULTS AND DISCUSSION

Chromatography

Sensitive, rapid, specific and reproducible HPLC method has been developed and validated for quantitative determination in Pioglitazone in rat serum samples. After the pretreatment with a rapid single liquid–liquid extraction step, the serum samples containing Pioglitazone were separated by reverse phase HPLC with UV detection at 269 nm

The representative chromatograms of Pioglitazone standard concentration and blank are shown Fig. 2 and Fig. 3. The retention time of Pioglitazone and Rosiglitazone were 7.07 min and 6.1 min respectively and the peaks were sharp. There was good baseline separation of Pioglitazone.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

Peak area ratio of Pioglitazone and Rosiglitazone were measured. A representative calibration graph of peak area versus concentration in the range of 0.1 µg/ml-10 µg/ml resulted in regression equation of the calibration curve was calculated as $y = 2.445x + 91.84$ (correlation coefficient, $r^2 = 0.9997$), where y is the peak area ratio of Pioglitazone and Rosiglitazone and x is the concentration of Pioglitazone. These results demonstrated a good linearity between the peak area ratios versus concentrations. The limit of detection (LOD) and limit of quantitation (LOQ) was 15ng/ml ($S/N \geq 3$)

and 50ng/ml. This method used a rapid single-step liquid-liquid extraction with ethyl acetate.

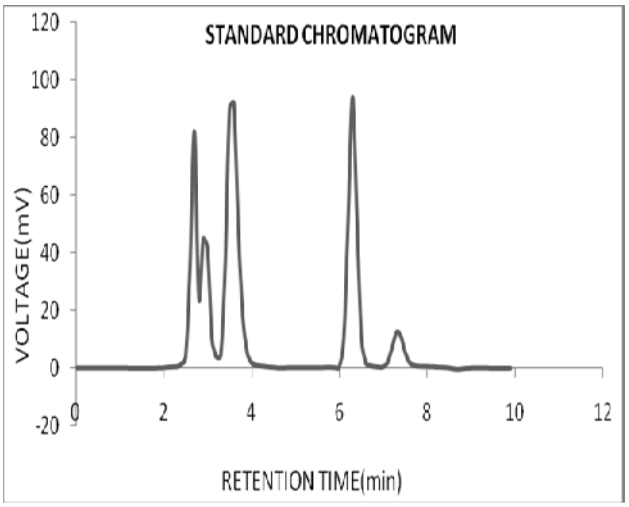

Fig. 2: Typical chromatogram of Pioglitazone 10µg/ml in serum (healthy rats)

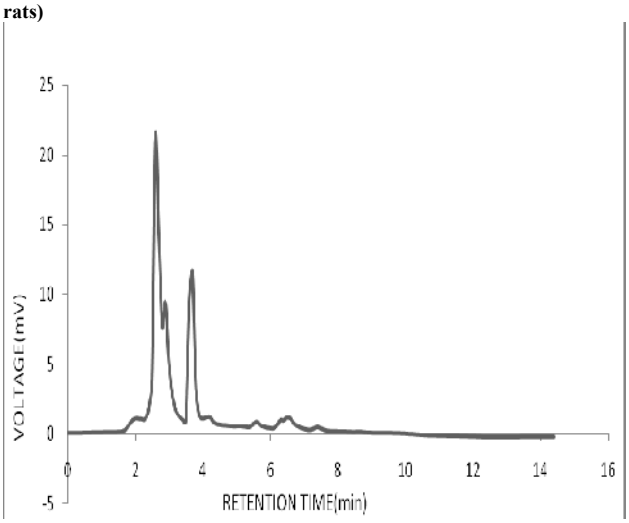

Fig. 3: Chromatogram of Blank serum

Table 1: Precision and accuracy of HPLC assay for Pioglitazone

Spiked concentrations (µg/ml)	Calculated concentration (µg/ml, mean± S.D, n=5)	RSD (%)	Deviation (%)
Intra-day (n=5)			
0.4	0.39±0.01	2.56	7.49
0.8	0.78±0.03	3.84	2.12
1	1.11±0.05	4.50	0.07
4	3.82±0.12	3.14	0.03
8	7.89±0.15	1.90	0.05
Inter-day (n=5)			
0.4	0.4±0.03	0.75	1.78
0.8	0.81±0.03	3.7	2.63
1	1.05±0.05	4.76	0.57
4	3.95±0.10	2.53	0.13
8	7.92±0.18	2.27	0.1

Precision and accuracy

The precision of the assay method was validated by the determination of the intra- and inter-day coefficient of variation (%RSD) and percentage deviation. The intra-day and inter-day precision has been done over the concentration range of 0.1µg/ml-10µg/ml. the average %RSD of intra-day and inter-day precision was 3.18% and 2.80% respectively. All %RSD are less than 5%. The accuracy of the method was verified by comparing the concentrations measured for Pioglitazone spiked from extracted sample with actual added concentrations. The intra- and inter-day accuracy data expressed as percentage deviation of Pioglitazone assay and the data was shown in Table 1.

Table 2: Recovery of pioglitazone from serum samples

QC samples (µg/ml)	Concentration of QC samples after extraction	Recovery (%)
0.4	0.387	96.7
0.8	0.785	98.1
1	0.971	97.1
4	3.860	96.5
8	7.78	97.2

Recovery

The recovery of Pioglitazone after liquid-liquid extraction procedures was evaluated at five concentrations of 0.4, 0.8, 1, 4, 8µg/ml. Absolute recovery was calculated by comparing the peak area ratios for direct injection of pure Pioglitazone and Rosiglitazone in methanol with those obtained by ethyl acetate extracted serum samples containing same amount of Pioglitazone and Rosiglitazone. Table 2 shows the recovery efficiency of Pioglitazone from rat serum samples and the average extraction efficiency of were found to 97.12%.

In summary, a method has been described for the determination of Pioglitazone in rat serum samples is sensitive, rapid, specific, accurate and reproducible. The excellent separation is demonstrated in the chromatograms and no interfering peaks were observed. The calibration curve was linear and the method was suitable for the analysis of serum samples over the range of 0.1 to 10µg/ml.

The differences of less than 5% for both the inter-day and intra-day data reflect the accuracy and precision of this method. A rapid single-step liquid-liquid extraction with ethyl acetate shows good recovery. The method described here is appropriate for a clinical study that does not require analysis of Pioglitazone metabolites and does not require a synthetic internal standard. A high-performance liquid chromatographic method for the determination of Pioglitazone in rat serum has been developed and validated. It has been shown to be accurate, precise and sensitive. This method can be used for pharmacokinetic studies of Pioglitazone.

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