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Liquid chromatographic method for the determination of rosiglitazone in human plasma

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Abstract

A robust, accurate and sensitive high-performance liquid chromatographic method for the determination of rosiglitazone (**I**) in human plasma has been developed. Pioglitazone (**II**) was used as internal standard. Both **I** and **II** are extracted from plasma using a liquid–liquid extraction procedure. Isocratic separation of **I** and **II** is carried out using a reversed-phase Zorbax SB C_{18}, 15-cm column with mobile phase consisting of methanol and a mixed phosphate buffer (10 m*M* monobasic sodium phosphate and dibasic sodium phosphate, pH adjusted to 2.6 with *ortho*-phosphoric acid) in the ratio 30:70 (v/v) and quantified by UV detection at 245 nm. Linearity was established over the range 5–1250 ng/ml using 1 ml human plasma. The method is specific, the endogenous components in plasma do not interfere with **I** and **II**. C.V. (%) of intra-day samples is less than 5.0% at four concentrations tested namely 5, 10, 500 and 1000 ng/ml. Similarly, over the same nominal concentrations, the precision of inter-day (5 days) samples also results in C.V. (%) less than 5.0%. The recoveries of **I** and **II** from human plasma were about 79 and 60%, respectively. This method can be used for routine clinical monitoring of **I**.

Keywords: Rosiglitazone

1. Introduction

Rosiglitazone (**I**), an aminopyridyl thiazolidinedione (Fig. 1), is a very potent synthetic peroxisome proliferator-activated receptor (PPAR)-γ agonist and effective antidiabetic agent [1,2]. It exerts its glucose-lowering effects by increasing insulin sensitivity in liver and peripheral tissues. Rosiglitazone has received regulatory approval for the treatment of type 2 diabetes as both monotherapy (USA) and in combination with oral antidiabetic agents (USA and Europe). The drug is highly bound to plasma proteins (99.8%) and is primarily eliminated via metabolism in the liver by cytochrome P450 isoenzyme 2C8. Following oral administration rosiglitazone is rapidly absorbed (T_{max} 1–2 h) with an elimination half-life of approximately 3–5 h [3–5]. Rosiglitazone has a pK_{a1} value of 6.08 and pK_{a2} of 6.80.

Literature survey reveals that **I** is estimated by HPLC methods. One method [6] involves the determination of **I** using automated sequential trace

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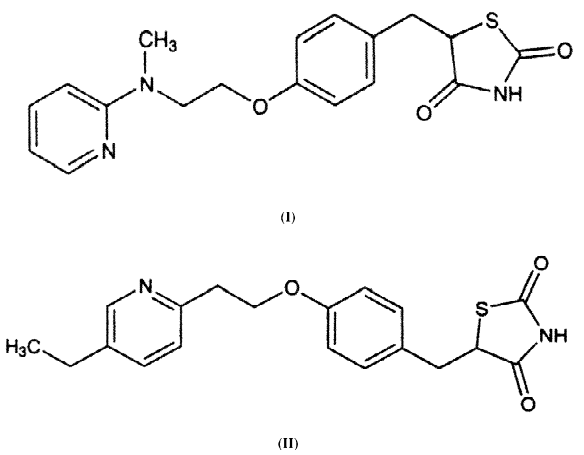

Fig. 1. Chemical structures for rosiglitazone (**I**, upper) and the internal standard, pioglitazone (**II**, lower) [11].

enrichment of dialysates (ASTED) coupled to reversed-phase HPLC with a fluorescence detector. Another method [7] involves isolation of the analyte through manual solid-phase extraction, followed by analysis with HPLC using a fluorescence detector. However, both the methods do not utilize liquid–liquid extraction and UV detection.

This paper describes the simple liquid–liquid extraction method for the determination of **I** in human plasma on reversed-phase HPLC with UV detection. This paper also describes the validation of the analytical procedure.

2. Experimental

2.1. Chemicals and reagents

Rosiglitazone and the internal standard pioglitazone (**II**; Fig. 1) were obtained from Wockhardt Research Centre (MIDC, Chikalthana, Aurangabad, Maharashtra State, India). All chemicals were obtained from Merck (Worli, Mumbai, Maharashtra State, India). Monobasic sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, hydrochloric acid, *ortho*-phosphoric acid and disodium tetraborate used were of A.R. grade. All solvents were obtained from Qualigens Fine Chemicals (Dr Annie Besant Road, Mumbai, Maharashtra State, India.). Methanol, acetonitrile, dichloromethane and *n*-hexane, used were of HPLC grade. Double distilled water was used throughout the procedure.

Monobasic potassium phosphate solution 0.001 *M* (pH 2.3) was prepared by dissolving 0.136 g of monobasic potassium phosphate in 800 ml of water and adjusting to pH 2.3 with 10% HCl (v/v) and diluting to 1000 ml with water. Disodium tetraborate solution (0.02 *M*; pH 9.3) was prepared by dissolving 7.6 g of disodium tetraborate in 1000 ml of water. Mixed phosphate buffer (10 m*M*; pH 2.6) was prepared by dissolving 1.41 g of dibasic sodium phosphate and 1.56 g of monobasic sodium phosphate in 800 ml water and adjusting pH 2.6 with *ortho*-phosphoric acid and diluting to 1000 ml with water.

2.2. Preparation of standard solutions and calibration standards

Stock solutions were prepared by dissolving 33.1 mg of rosiglitazone maleate (equivalent to 25 mg of the rosiglitazone base) in acetonitrile in a 100-ml volumetric flask to yield primary solutions with a concentration of 250 μg/ml of **I** (free base). Secondary and working standard solutions were prepared by dilution with the buffer solution pH 2.3. Calibration standards were prepared by spiking working standard solutions into drug-free plasma to yield concentrations of 5, 10, 25, 50, 100, 250, 500, 750, 1000 and 1250 ng/ml of **I**.

Internal standard stock solution was prepared by dissolving 25 mg of **II** in acetonitrile in a 100-ml volumetric flask. This was further diluted with the buffer solution pH 2.3 to yield 10 μg/ml **II**.

2.3. Instrumentation

The HPLC system consisted of a HPLC pump (Model Constametric 3500 Thermo Separation Products), an autosampler (Model AS 3000 Thermo Separation Products) and UV variable wavelength detector (Model UV 1000 Thermo Separation Products) set at 245 nm, an interface (Model SP4510, Thermo Separation Products) and a datastation with PC 1000 software version 3.5.1.

2.4. Extraction procedure

To 1 ml plasma in a test-tube, 50 μl of **I** and **II** were added and the tube was vortexed for 1 min, 200 μl disodium tetraborate solution was then added, and the tube again vortexed for 1 min. To this 5 ml of dichloromethane was added and the tube shaken horizontally for 10 min. The tube was then centrifuged at 735 *g* for 10 min at room temperature. The organic layer was then removed and evaporated to dryness under a stream of nitrogen at ambient temperature using a Zymark Turbo Evaporator. To the residue, 5 ml of a solvent mixture, *n*-hexane–dichloromethane (80:20, v/v) was added and the tube was vortexed for 30 s. Then 350 μl of buffer solution pH 2.3 was added to the tube and the solution was vortexed for 2 min, and centrifuged at 735 *g* for 10 min. After phase separation by centrifugation and subsequent removal of the top organic layer, the aqueous phase containing the extracted analytes was ready for analysis by HPLC.

2.5. HPLC conditions

The chromatographic analysis was carried out on a Zorbax SB C_{18} column (5-μm particle size, 150 mm×4.6 mm I.D.) maintained at 30 °C. **I** and **II** were eluted using a mobile phase composition of 10 m*M* mixed phosphate buffer pH 2.6 and methanol (70:30, v/v) at a flow-rate of 1.2 ml/min. The mobile phase was premixed, filtered through a 0.45 μm Nylon 66 membrane filter and degassed before use. Nylon 66 membrane filter papers were obtained from Advanced Microdevices Pvt. Ltd. (Ambala Cantt, India). Typical retention times for **I** and **II** were 8.3 and 18.0 min, respectively (Fig. 2c). Injection volume of the samples was 100 μl. The peaks were determined using a UV detector set at wavelength 245 nm.

2.6. Specificity and linearity

The specificity of the method was evaluated with regard to interference due to the presence of endogenous substances in the extracted human plasma. For the same, plasma pouches were randomly chosen for experimentation. Six different plasma samples were extracted and injected.

To establish the range of linearity between the plasma drug concentration and detector response, plasma drug concentrations of 5, 10, 25, 50, 100, 250, 500, 750, 1000 and 1250 ng/ml were used.

2.7. Limit of quantification

Limit of quantification was defined as the lowest concentration at which the precision expressed by relative standard deviation (RSD) is less than 20% and accuracy expressed by relative difference in the measured and true value was also less than 20%.

To determine the limit of quantification, plasma solutions containing 1, 2, 3, 4, 5, 6, 10, 25, 50, 100, 250, 500, 750, 1000 and 1250 ng/ml of **I** were prepared and extracted as per the procedure and injected into the chromatographic system. The values were back-calculated from the standard curve.

2.8. Recovery

The recoveries of **I** and **II** in the extraction procedure were determined by comparing the peak areas obtained from an extracted sample spiked with known amounts of **I** and **II** with those obtained from the pure compounds of the same concentrations in the solutions. The recoveries were determined in triplicate at three concentrations 10, 500, 1000 ng/ml for **I** and 500 ng/ml for **II**.

2.9. Precision and accuracy

To assess the precision of the method, intra-day and inter-day (days 1, 2, 3, 4 and 5) measurements of rosiglitazone were completed with computation of the coefficient of variation (C.V.%) for replicate samples ($n=5$ for intra-day, $n=25$ for inter-day) using concentrations of 5, 10, 500 and 1000 ng/ml. Both intra-day and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis.

Accuracy (expressed as % bias) was calculated as the percent difference between the amount of **I** added and found.

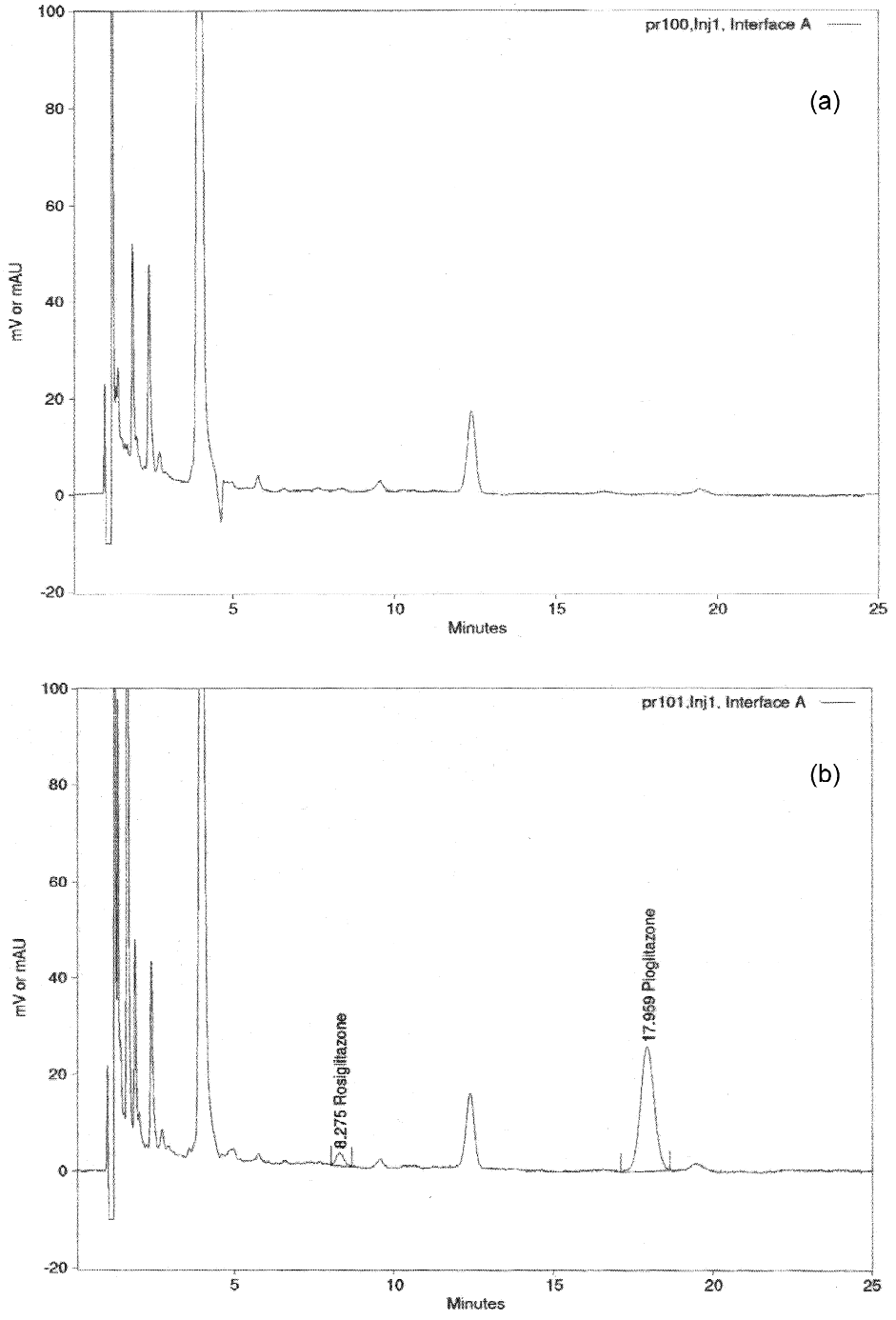

Fig. 2. (a) HPLC chromatogram of blank plasma sample; (b) HPLC chromatogram of plasma spiked with 5 ng/ml of rosiglitazone (LOQ) and 500 ng/ml of internal standard, pioglitazone; (c) HPLC chromatogram of plasma spiked with 100 ng/ml of rosiglitazone and 500 ng/ml of internal standard, pioglitazone.

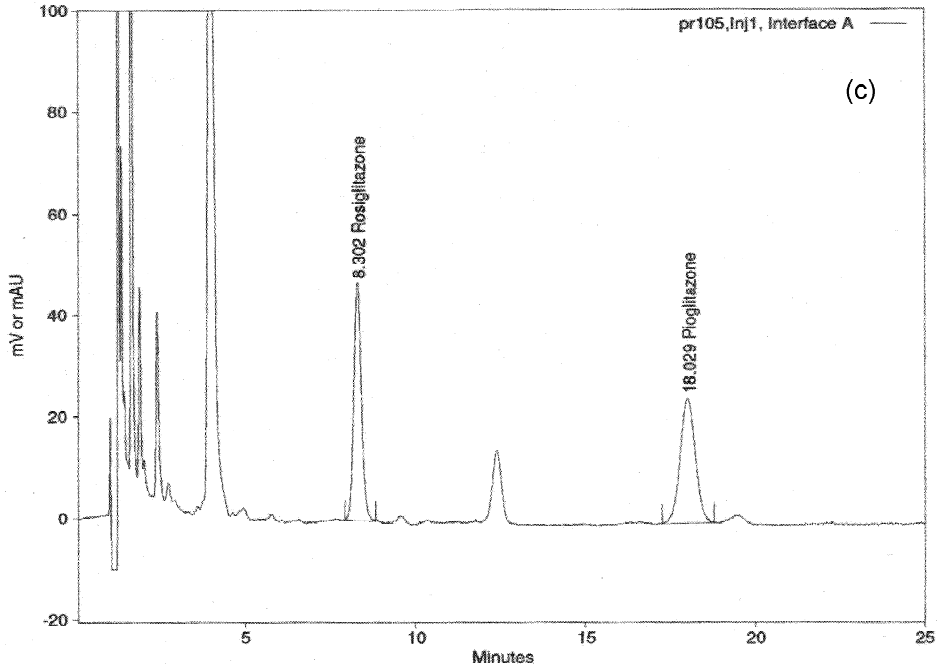

Fig. 2. (*continued*)

3. Results

3.1. Specificity and linearity

Fig. 2a–c illustrates HPLC chromatograms of blank plasma sample; plasma spiked with 5 ng/ml of rosiglitazone (LOQ) and 500 ng/ml of the internal standard pioglitazone; and plasma spiked with 100 ng/ml of rosiglitazone and 500 ng/ml of the internal standard pioglitazone. The chromatogram of blank plasma does not show any interference of plasma peaks. The total eluting time was less than 25 min. The regression lines relating standard concentrations of **I** and peak area ratios were calculated using weighted regression analysis [weight=1/(concentration)^2] [8,9]. The calibration curves were linear in the studied range.

The mean±standard deviation (SD) for the slope, intercept and correlation coefficient of the standard curves (n=5) are 0.00935±0.00022, 0.00226±0.00967 and 0.9996±0.00025, respectively.

3.2. Limit of quantification

It was found that below 5 ng/ml, the back-calculated values failed to meet the acceptance criteria. Hence the 5 ng/ml level was extracted five times and injected. It was found that the relative standard deviation was 3.25% and accuracy, defined as the deviation between the true value and the measured value expressed as a percentage was 4.0% at this concentration. So 5 ng/ml was established as the lowest limit of quantification.

3.3. Recovery

Percent recoveries of compounds from spiked plasma following the extraction procedure were found and are represented as mean±standard deviation. For **I**, at concentrations 10, 500 and 1000 ng/ml, the recoveries were 81±2.41, 79±1.70 and 76±5.74, respectively. For **II**, at a concentration of 500 ng/ml, the recovery was 60±1.94.

Table 1
Intra-assay precision and accuracy

n	Conc. added (ng/ml)	Conc. found (ng/ml)	Bias (%)	RSD (%)
5	5	5.20	4.00	3.25
5	10	10.10	1.00	4.17
5	500	497.55	−0.49	1.98
5	1000	1008.42	0.84	1.78

3.4. Precision and accuracy

Intra-assay precision of the method is illustrated in Table 1. This was estimated by assaying the quality control samples (5, 10, 500 and 1000 ng/ml) five times in the same analytical run. The precision was less than 5% and the percentage bias was less than 5% at all levels.

Inter-assay precision of the method is illustrated in Table 2. This was estimated by assaying the quality control samples (5, 10, 500, 1000 ng/ml) for replicate samples ($n=25$). The precision was less than 5% and the percentage bias (accuracy) was less than 5% at all levels meeting the acceptance criteria of ±20% at LOQ and ±15% at other levels.

3.5. Stability study

3.5.1. Freeze-thaw stability

Freeze-thaw stability was carried out at three different concentrations (10, 500 and 1000 ng/ml). The plasma samples were stored at −40 °C and subjected to three freeze–thaw cycles. The results are represented in ng/ml from initial to after the third cycle as (mean±standard deviation). For concentrations of 10, 500 and 1000 ng/ml, the results found were 9.71±0.26, 511.89±13.41 and 1017.46±10.63, respectively. The concentrations found are well within the acceptable limit ±15% of the nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

Table 2
Inter-assay precision and accuracy

n	Conc. added (ng/ml)	Conc. found (ng/ml)	Bias (%)	RSD (%)
25	5	5.20	4.0	3.30
25	10	9.94	−0.6	3.94
25	500	495.02	−1.0	2.21
25	1000	1005.88	0.59	3.25

3.5.2. Processed sample stability

Processed sample stability was carried out at three different concentrations, 10, 500 and 1000 ng/ml. The samples were kept in an autosampler at ambient temperature and analysed at regular intervals up to 72 h. The results are presented in Table 3. The processed samples were found to be stable at ambient temperature for 72 h.

3.5.3. Bench top stability

Bench top stability was carried out at three different concentrations (10, 500 and 1000 ng/ml). The plasma samples were kept at ambient temperature and analysed up to 180 min. The results are shown in Table 4. The concentrations found are well within the acceptable limit ±15% of the nominal concentration.

4. Discussion

The chromatographic method was optimised by changing various parameters, such as pH of the mobile phase, organic modifier and buffer concentration. The effect of pH of the buffer used in the mobile phase was more pronounced on the retention of **II** than **I**. The retention time of **II** increased with the increase in the pH of the buffer. The separation of the peaks due to plasma and **I**, **II** is dependent on the pH of the buffer and the percentage of methanol

Table 3
Processed sample stability

Sample conc. (ng/ml)	Initial		After 24 h		After 48 h		After 72 h	
	Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)
10	10.74	7.40	10.48	4.80	9.78	−2.20	10.40	4.00
500	520.39	4.08	536.65	7.33	515.08	3.02	511.05	2.21
1000	987.78	−1.22	998.88	−0.11	981.21	−1.88	968.05	−3.20

Table 4
Bench top stability

Sample conc. (ng/ml)	n	Initial		After 60 min		After 120 min		After 180 min	
		Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)
10	3	10.18	1.80	10.31	3.10	10.25	2.50	10.41	4.10
500	3	500.72	0.14	503.93	0.79	492.69	−1.46	492.28	−1.54
1000	3	925.20	−7.48	935.61	−6.44	927.41	−7.26	923.29	−7.67

in the mobile phase. The peaks due to plasma in close proximity to **I** and **II** could be resolved by decreasing the pH of the buffer.

Acetonitrile was used instead of methanol in the mobile phase to shorten the retention time of **I** and **II**, but it was observed that the plasma peaks interfered with **I** and **II**.

Under the presently prescribed conditions for extraction of **I** and **II**, the recoveries were found to be 79 and 60%, respectively. The recovery of **II** was less compared to the recovery of **I**. This was because of its relatively higher solubility in the organic solvent mixture (*n*-hexane–dichloromethane, 80:20, v/v) than **I**. The percentage of *n*-hexane was increased to increase the recovery of **I** and **II** but it also resulted in increasing the endogenous interfering substances that were not seen under the present conditions. A very low concentration buffer (0.001 *M* monobasic potassium phosphate, pH adjusted to 2.3 with hydrochloric acid) was used for the back-extraction of **I** and **II** to reduce the amount of co-extracting endogenous interfering substances. Another benefit of using this buffer was the stability of **I** and **II** in it. It is necessary to maintain the pH of the buffer to 2.3 because as the pH of the buffer increases, the solubility of **I** decreases.

The previously published two methods [6,7] consist of automated techniques for sample extraction and a specific fluorescence detector. These techniques are expensive and would not be available in every laboratory. Considering the availability of the automated instruments and the cost involved, we developed a new method which includes a liquid–liquid extraction procedure for the estimation of **I** in human plasma using a universal UV detector.

In the previous method [6], a Millipore column was used for the estimation of **I**. The pH of the mobile phase was 8.0, which was beyond the limit (pH 3.5–6.5) given by the column manufacturers (Millipore) for column longevity. In the present method, a Zorbax column has been used and the buffer pH in the mobile phase is 2.6 which is within the limits (pH 2–8) specified by the manufacturers (Zorbax).

The differences of less than 5% for both the inter-day and intra-day data reflect the accuracy of this method. The observations of C.V. less than 5% for both inter-day and intra-day measurements also indicate a high degree of precision. In the previous method, within and between run precision and accuracy of determination were better than 10% across the calibration range.

Currently, rosiglitazone is available as 2-, 4- and 8-mg tablets. The C_{max} values for the 2-mg tablet is 156 ng/ml and for the 8-mg tablet is 598 ng/ml [10]. In previously published methods [6,7] the linearity has been shown up to 100 ng/ml. But they found that when the 8-mg dose was administered to the subjects, the plasma levels of **I** were above 100 ng/ml. The samples had to be therefore diluted and reanalysed. Subsequently, the method was revalidated over an extended linearity range of 5–1000 ng/ml [6].

In the present method, we have established a linearity range of 5–1250 ng/ml. This linearity range covers the C_{max} values of all the three strengths of rosiglitazone. Hence this method can be applied for quantifying the levels of **I** in vivo when either of the dosages is administered to the subjects.

4.1. Application

The application of the method for the assay of **I** in human plasma has been shown by orally administering a 4-mg tablet of rosiglitazone to the subject. The subject plasma was processed and analysed as per the analytical method. A representative chromatogram from a clinical subject administered **I** orally is shown in Fig. 3.

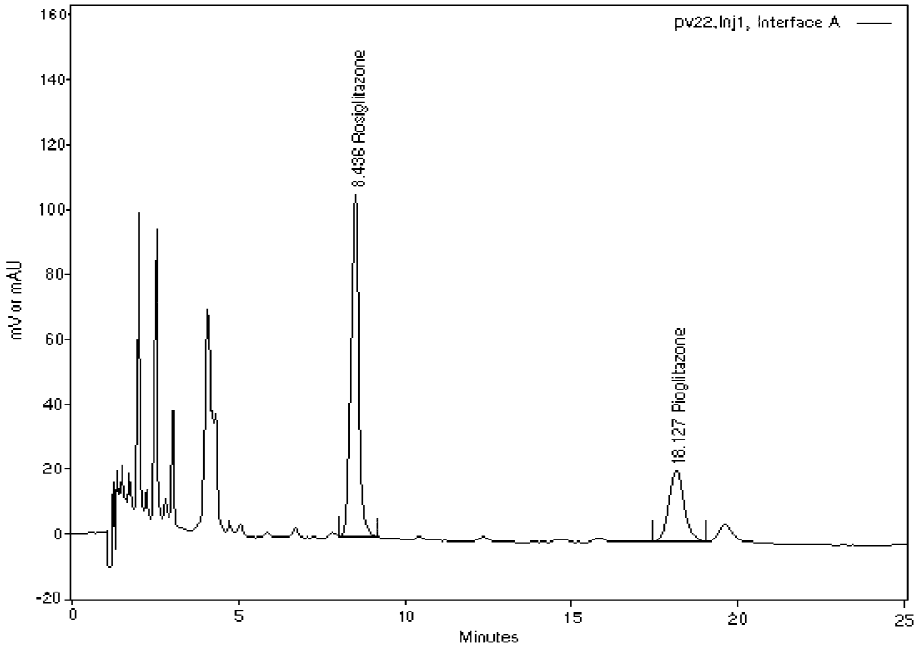

Fig. 3. HPLC chromatogram of a clinical plasma sample taken from a volunteer 20 min after a 4-mg oral dose of rosiglitazone tablet (quantitated to be 261.6 ng/ml).

4.2. Conclusion

A high-performance liquid chromatographic method for the determination of rosiglitazone (**I**) in human plasma has been developed. It has been shown to be accurate, precise and sensitive. There was no evidence of instability of **I** in human plasma following three freeze–thaw cycles. Also **I** showed bench top stability up to 3 h and the processed samples of **I** were found to be stable for 72 h. The method can be used for pharmacokinetic studies of **I**.

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