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High-performance liquid chromatographic method for the determination of pioglitazone in human plasma using ultraviolet detection and its application to a pharmacokinetic study

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Abstract

An analytical method based on high-performance liquid chromatography (HPLC) with ultraviolet detection (269 nm) was developed for the determination of pioglitazone in human plasma. Rosiglitazone was used as an internal standard. Chromatographic separation was achieved with a reversed-phase Apollo C18 column and a mobile phase of methanol–acetonitrile-mixed phosphate buffer (pH 2.6; 10 mM) (40:12:48, v/v/v) with a flow rate of 1.2 ml/min. The calibration curve was linear over the range of 50–2000 ng/ml ($r^2 > 0.9987$) and the lower limit of quantification was 50 ng/ml. The method was validated with excellent sensitivity, accuracy, precision, recovery and stability. The assay has been applied successfully to a pharmacokinetic study with human volunteers.

Keywords: Pioglitazone; HPLC; Pharmacokinetics; Quantification; Validation; Solid-phase extraction; Reversed-phase chromatography

1. Introduction

Pioglitazone ([(±)-5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy] phenyl]methyl]-2,4-] thiazolidinedione) hydrochloride (Fig. 1) is an oral anti-hyperglycemic agent that acts primarily by increasing insulin sensitivity in target tissues. 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) [1–3]. Several liquid chromatography methods have been described in the literature to determine pioglitazone and its metabolites in biological fluids [4–8]. The classical procedure used was high-performance liquid chromatography with ultraviolet detection (HPLC-UV) with run times of more than 20 min [5,6]. Liquid chromatography with tandem mass spectroscopy (LC–MS/MS) has been performed with a lower limit of quantification (LLOQ) of 0.5 ng/ml [7,8]. The sample preparation techniques were based on either liquid–liquid extraction or solid-phase extraction (SPE), and often depended on the type of detector used [4–8]. A drawback of these methods was the use of a synthesized internal standard [5–7], which is not readily available. Additionally, the method of Xue et al. [8] utilized a commercially available rosiglitazone (Fig. 2), another thiazolidinedione anti-hyperglycemic agent. The method described here has been developed due to the expense of LC–MS/MS instruments, and because pioglitazone metabolites are not necessary for the measurement.

We describe the validation of a method to measure pioglitazone in human plasma. The method uses HPLC-UV with rosiglitazone as the internal standard. The method exhibited sufficient levels of sensitivity, selectivity, accuracy and precision for a pharmacokinetic study within the FDA guidelines [9]. A summary of the pharmacokinetic results of pioglitazone from Thai healthy male volunteers is presented.

2. Experimental

2.1. Chemicals and reagents

Pioglitazone hydrochloride (>99% purity) for use as a reference standard and rosiglitazone (>99% purity) for use as an

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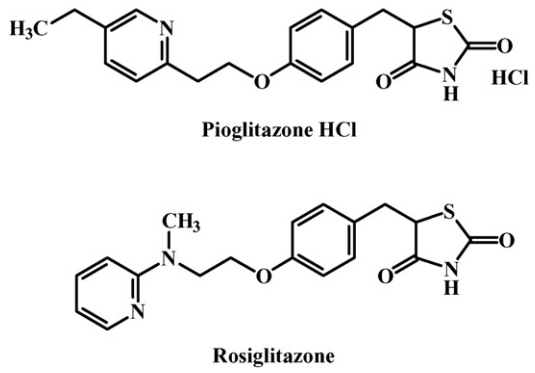

Fig. 1. Chemical structures of pioglitazone and rosiglitazone.

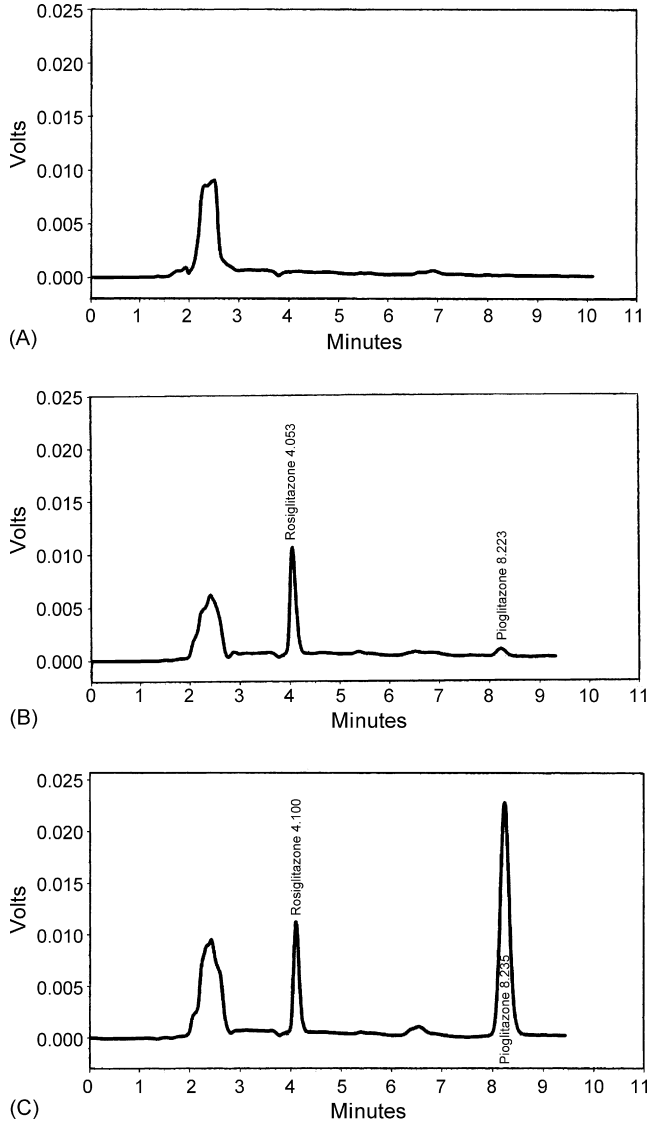

Fig. 2. Representative HPLC chromatograms for the analysis of pioglitazone in drug-free human plasma: (A) blank plasma (B) human plasma spiked with 50 ng/ml of pioglitazone and 50 μg/ml of rosiglitazone and (C) human plasma spiked with 2000 ng/ml of pioglitazone and 50 μg/ml of rosiglitazone.

internal standard were purchased from Crosschem Intercontinental Company, Derb & Co. (Lugano, Switzerland). Sodium dihydrogen phosphate, disodium hydrogen phosphate and *ortho*-phosphoric acid were of analytical reagent grade. All solvents were of HPLC grade and were obtained from VWR Prolabo (VWR International Ltd., Dorset, England) and the other chemicals were of analytical reagent grade. Mixed phosphate buffer (pH 2.6; 10 mM) was prepared by dissolving 1.36 g of potassium dihydrogen phosphate (KH_2PO_4) and 1.74 g of dipotassium hydrogen phosphate (K_2HPO_4) in 800 ml of water, adjusting to pH 2.6 with *ortho*-phosphoric acid and diluting to 1000 ml. Purified water was obtained from TKA ROS 300 (Niederelbert, Germany). Human plasma samples were provided by Naresuan University Hospital (Phitsanulok, Thailand).

2.2. Chromatographic conditions

The HPLC system consisted of a dual plunger pump (LC-10ATVP, Shimadzu, Kyoto, Japan), a UV–vis detector (SPD-10AVP, Shimadzu) equipped with a system controller (SCL-10AVP, Shimadzu) and a Rheodyne (7725) sample injector (Rohnert Park, CA) fitted with a 100-μl sample loop. The chromatographic separations were carried out on Apollo C18 column (250 mm × 4.6 mm i.d., 5 μm, 250 Å from Alltech, Deerfield, IL) fitted with a refillable guard cartridge packed with Apollo C18 (7.5 mm × 4.6 mm i.d., 5 μm, Alltech). The mobile phase was methanol–acetonitrile-mixed phosphate buffer (pH 2.6; 10 mM) (40:12:48, v/v/v). All separations were performed isocratically at a flow-rate of 1.2 ml/min. Column temperature was maintained at room temperature (27 ±2 °C) and the temperature of the column had an effect only on the retention of analytes. The peaks were determined using a UV detector set at a wavelength of 269 nm based on Zhong and Williams [5] and Yamashita et al. [6]. All the procedures were performed at room temperature.

2.3. Preparation of stock and standard solutions

The stock solutions of pioglitazone and the internal standard were prepared separately in acetonitrile to yield primary standard solutions with a concentration of 1 mg/ml as the base. Secondary standard solutions of pioglitazone were prepared by dilution with the mobile phase. Working standard solutions of pioglitazone were prepared by diluting the secondary standard solutions with drug-free human plasma, giving final concentrations of 50, 100, 200, 500, 1000 and 2000 ng/ml. The working internal standard solution with a concentration of 50 μg/ml was prepared by diluting the stock solution with the mobile phase. The quality control (QC) samples at a concentration of 75 ng/ml (low-quality control, LQC sample) and 1500 ng/ml (high-quality control, HQC sample) were prepared by dilution of the secondary standard solution with drug-free human plasma.

2.4. Preparation of samples

The solid-phase extraction column (SPE, Strata C18-T, Phenomenex, Torrance, CA) was pre-activated with 1 ml each of acetonitrile and KH_2PO_4 (0.1 M). Then 70 μl of the internal

standard solution (50 μg/ml) and 500 μl of KH_2PO_4 (0.1 M) were added to a 1-ml aliquot of working pioglitazone solution in plasma. The tubes were vortex-mixed briefly (Genie 2, Scientific Industries Inc., Bohemia, NY). The mixture was applied to the activated SPE column. The column was washed with 2 ml of methanol–KH_2PO_4 (0.1 M) (30:70, v/v) followed by 1 ml of K_2HPO_4 (0.1 M) and was then dried for 5 min. The analytes were eluted with 500 μl of acetonitrile–H_2O (40:60, v/v) followed by 500 μl of acetonitrile–H_2O (50:50, v/v). The total eluate was filtered through a 0.45-μm pore size nylon disposable syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) and 100 μl was injected into the HPLC system for analysis.

2.5. Bioanalytical method validation

2.5.1. Calibration curve

The linearity of the method was evaluated by a calibration curve in the range of 50–2000 ng/ml of pioglitazone, including LLOQ. The calibration curve was obtained by plotting the area ratios of pioglitazone and internal standard versus the concentration of pioglitazone by least-squares linear regression analysis. The calibration curve requires a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration should be within 15% of the nominal value, except it should not exceed 20% at the LLOQ.

2.5.2. Accuracy and precision

Intra-day and inter-day accuracy and precision were determined by replicate analysis of six sets of samples spiked with six different concentrations of pioglitazone (50, 100, 200, 500, 1000 and 2000 ng/ml) within a day or on 6 consecutive days. For acceptable intra-day and inter-day values, accuracy should be within 85–115% and coefficient of variation (CV) values should be <15% over the calibration range, except at the LLOQ, where accuracy should be between 80 and 120% and CV should not exceed 20%.

2.5.3. Recovery

The recovery of pioglitazone from plasma was determined at concentration ranges of 50, 100, 200, 500, 1000 and 2000 ng/ml by comparing each peak area of six extracted samples with mean peak areas of six unextracted standard solutions containing the corresponding concentrations in the mobile phase that represent 100% recovery. The recovery of internal standard from plasma was determined at a concentration of 50 μg/ml by the same method. Recovery of the analytes need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible.

2.5.4. Stability

Analyte stability in plasma was tested using low-quality and high-quality control samples for three freeze–thaws, long-term, short-term and post-preparative stabilities. The freeze–thaw stability of the analyte was determined over three freeze–thaw cycles within 3 days. In each freeze–thaw cycle, the spiked plasma samples were frozen for ~24 h at −80 °C and thawed at room temperature. The long-term stability was evaluated after keeping the plasma samples frozen at −80 °C for 2 months. For the short-term stability, frozen plasma samples were kept at room temperature for 6 h before sample preparation. The stability of the prepared plasma samples was tested after keeping the samples at room temperature for 24 h. The samples were analyzed and the results were compared with those obtained for freshly prepared samples. For the acceptance criterion of stability, the deviation compared to the freshly prepared standard should be within ±15%.

2.6. Pharmacokinetic application

The validated method was applied to a pharmacokinetics study of pioglitazone in 24 healthy Thai male volunteers with a mean age of (21.3 ± 1.37) years and a mean body mass index (BMI) of 20.8 ± 2.60, after oral administration of a 30-mg pioglitazone tablet (Glubosil®, Silom Medical, Co. Ltd., Bangkok, Thailand). The volunteers fasted overnight before the study and for 4 h after the dosing. Venous blood samples (8 ml) were collected 0.5 h before dosing and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 48 and 72 h following administration. The blood samples were centrifuged at 5000 rpm for 15 min at 4 °C and the plasma was removed and stored in a cryogenic vial (Nalge Nunc International, Rochester, NY, USA) at −80 °C. The pharmacokinetic parameters for each volunteer were evaluated from the plasma concentration–time profile calculated by WinNonlin Professional version 4.0.1 (Pharsight Corporation, Mountain View, CA, USA) with non-compartment analysis. This study was approved by the Naresuan University Ethical Committee before obtaining informed written consent from all volunteers.

3. Results and discussion

3.1. Specificity and optimization of the chromatographic conditions

To serve as blanks, plasma samples were obtained from six different sources and assayed to evaluate the selectivity of the method and the detection of interference. Representative chromatograms of human blank plasma; human blank plasma spiked with 50 ng/ml of pioglitazone (LLOQ) and 50 μg/ml of the internal standard; and human blank plasma spiked with 2000 ng/ml of pioglitazone and 50 μg/ml of the internal standard are shown in Fig. 2A–C, respectively. The peaks of internal standard and pioglitazone were well resolved, with retention times of 4.1 and 8.2 min, respectively. No endogenous peak from plasma was found to interfere with the elution of either the drug or the internal standard. Analysis could be achieved within 10 min for a total chromatography run. On the basis of our results, the total run time was less than that reported by Zhong and Williams [5] and Yamashita et al. [6] using ultraviolet detection. Furthermore, this simple method did not require programming the flow rate [5] or a gradient of the mobile phase [6]. Column clean-up by the combination of solid-phase and liquid–liquid extraction to eliminate interferences [6] was not necessary in this study. Cleansing of the column with K_2HPO_4 (0.1 M) after everyday use was necessary for long-term efficiency and to avoid broad-

Table 1
Linearity obtained after least-squares regression analysis of the method for determining pioglitazone in plasma samples

Number	Slope	Intercept	r^2
1	0.0017	0.0206	0.9994
2	0.0018	−0.0125	0.9998
3	0.0018	0.0024	0.9990
4	0.0018	−0.0127	0.9998
5	0.0018	−0.0282	0.9996
6	0.0018	−0.0275	0.9997
Mean	0.0018	−0.0096	0.9996
S.D.	0.0000	0.0187	0.0003
S.E.	0.0000	0.0076	0.0001

Six calibration standards were included in each calibration curve. S.D., standard deviation; S.E., standard error.

ened peaks of both drug and internal standard. Replacement of potassium with sodium salts of both phosphate buffers in all SPE processes was found to affect both the retention time and the peak shape of pioglitazone and the internal standard significantly. The SPE of the samples can be performed either the day before analysis or on the day of analysis. A ghost sharp peak with high intensity occurred between the internal standard and the drug peaks when the eluate was filtered through a different brand of nylon filter syringe (Titan®, Sun SRi, Rockwood, TN, USA). No peak of pioglitazone metabolites was seen in the chromatogram.

3.2. Linearity and sensitivity

The calibration curve was linear over the pioglitazone concentration range 50–2000 ng/ml in human plasma. Table 1 shows the individual calibration equations of pioglitazone from six replicate experiments. The equation of the curve, obtained by a least-squares method, was $y = 0.0018x–0.0096$ (where y is the peak area ratio of the analyte to internal standard and x is the concentration of the analyte). The correlation coefficient (r^2) of the calibration curve generated during the validation was 0.9996 for the analyte. Table 2 summarizes the accuracy and precision of the calibration curve. The LLOQ of pioglitazone in plasma was verified as 50 ng/ml, as this was the lowest concentration assessed at which the accuracy was between 80 and 120%, and precision was within 20%. The lower limit of detection (LOD) was 25 ng/ml at a signal-to-noise ratio of 3.

Table 2
Accuracy and precision of calibration standards of the method for determining the concentration of pioglitazone in plasma samples ($n = 6$)

Known concentration (ng/ml)	Concentration found (mean ± S.D.; ng/ml)	Accuracy (%)	Precision (%CV)
50	47.91 ± 3.19	95.8	6.7
100	94.86 ± 5.87	94.9	6.2
200	207.17 ± 6.23	103.6	3.0
500	492.55 ± 16.86	98.5	3.4
1000	1010.08 ± 27.33	101.0	2.7
2000	1994.29 ± 54.13	99.7	2.7

Table 3
Accuracy and precision of the method for determining the concentration of pioglitazone in plasma samples ($n = 6$)

Known concentration (ng/ml)	Concentration found (Mean ± S.D.; ng/ml)	Accuracy (%Accuracy)	Precision (%CV)
Intra-day			
50	48.21 ± 3.81	96.4	7.9
100	94.88 ± 8.28	94.9	8.7
200	189.43 ± 8.53	94.7	4.5
500	474.72 ± 16.57	94.9	3.5
1000	956.25 ± 19.48	95.6	2.0
2000	1951.52 ± 69.91	97.6	3.6
Inter-day			
50	47.62 ± 2.47	95.2	5.2
100	96.02 ± 6.54	96.0	6.8
200	182.98 ± 6.49	91.5	3.6
500	460.66 ± 12.40	92.1	2.7
1000	902.89 ± 7.86	90.3	0.9
2000	1909.62 ± 86.66	95.5	4.5

3.3. Accuracy and precision

The results for accuracy and precision at concentrations of 50–2000 ng/ml for pioglitazone are presented in Table 3. The intra-day accuracy and precision varied between 94.7 and 97.6%, and between 2.0 and 8.7%, respectively. The inter-day accuracy and precision ranged from 90.3 to 96.0% and from 0.9 to 6.8, respectively. All the values of accuracy and precision including LLOQ fell within the limits considered as acceptable.

3.4. Recovery

The recovery of pioglitazone in the SPE procedure from 1 ml of plasma was measured at six different concentrations over the calibration range used. Table 4 shows the absolute recovery, expressed as a percentage, obtained for both pioglitazone and the internal standard. Regardless of the drug concentration, the recoveries ranged from 96.6 to 106.3% with a CV between 2.3 and 7.1%. No clear relationship between concentration and recovery was found. A recovery of 98.8% was obtained for the internal standard. The pioglitazone recovered from the sample

Table 4
Absolute recovery of the method for determining the concentration of pioglitazone in plasma samples

Concentration	Absolute recovery (mean ± S.D.; %)	%CV
Pioglitazone[a] (ng/ml)		
50	101.4 ± 6.5	6.4
100	101.0 ± 2.3	2.3
200	96.6 ± 6.8	7.1
500	106.3 ± 2.8	2.6
1000	101.8 ± 6.5	6.4
2000	100.4 ± 4.8	4.8
Rosiglitazone[b] (μg/ml)		
50	98.8 ± 5.7	5.7

[a] $n = 6$.
[b] $n = 36$.

Table 5
Stability of pioglitazone in human plasma

Stability ($n=3$)	Concentration (mean ± S.D.; ng/ml)	
	75[a]	1500[b]
Freeze–thaw stability		
Initial	74.4 ± 2.4	1496.6 ± 56.6
Measured	73.5 ± 1.1	1366.9 ± 13.8
Deviation (%)	−1.2	−8.7
Long-term stability		
Initial	75.1 ± 1.4	1522.7 ± 17.6
Measured	75.4 ± 0.6	1521.6 ± 10.2
Deviation (%)	+0.3	−0.1
Short-term stability		
Initial	73.8 ± 2.5	1538.1 ± 18.1
Measured	74.3 ± 2.7	1500.5 ± 23.1
Deviation (%)	+0.7	−2.5
Post-preparative stability		
Initial	73.9 ± 6.0	1443.7 ± 4.8
Measured	77.5 ± 2.8	1454.8 ± 8.6
Deviation (%)	+4.9	+0.8

[a] Low-quality control samples.
[b] High-quality control samples.

plasma was greater than 100%, which may be caused by errors in the two pipetting steps in the SPE process. The major analytes from SPE eluted sequentially by acetonitrile–H_2O (40:60) and acetonitrile–H_2O (50:50) as the solvent systems were drug and internal standard, respectively. With the SPE procedure used by Zhong and Williams [5], the rosiglitazone internal standard could not be eluted totally from the column and some interfering compounds were present with the analytes.

3.5. Stability

Plasma samples of pioglitazone at two concentrations (75 and 1500 ng/ml) were used for stability experiments. Stability was assessed under a variety of conditions and the maximum period of confirmed stability is presented in Table 5. The deviation of the mean test responses were within ±15% of appropriate controls in all stability tests of pioglitazone in human plasma. Three freeze–thaw cycles of the quality control samples appeared to have no effect on quantification of the analyte. Quality control samples stored in a freezer at −80 °C remained stable for at least 2 months. No effect on quantification was observed for the short-term stability of the frozen samples kept at room temperature for 6 h. The extracted samples were analyzed after at least 24 h at room temperature. These studies suggested that human plasma samples containing pioglitazone can be handled under normal laboratory conditions without significant loss of compound.

3.6. Pharmacokinetic application

The validated method has been used successfully to quantify the concentration of pioglitazone in human plasma samples after the administration of a single 30 mg oral dose of pioglitazone. Fig. 3 shows the chromatograms of plasma samples obtained from a volunteer at 0, 1.5 and 8 h after dosing. The mean plasma

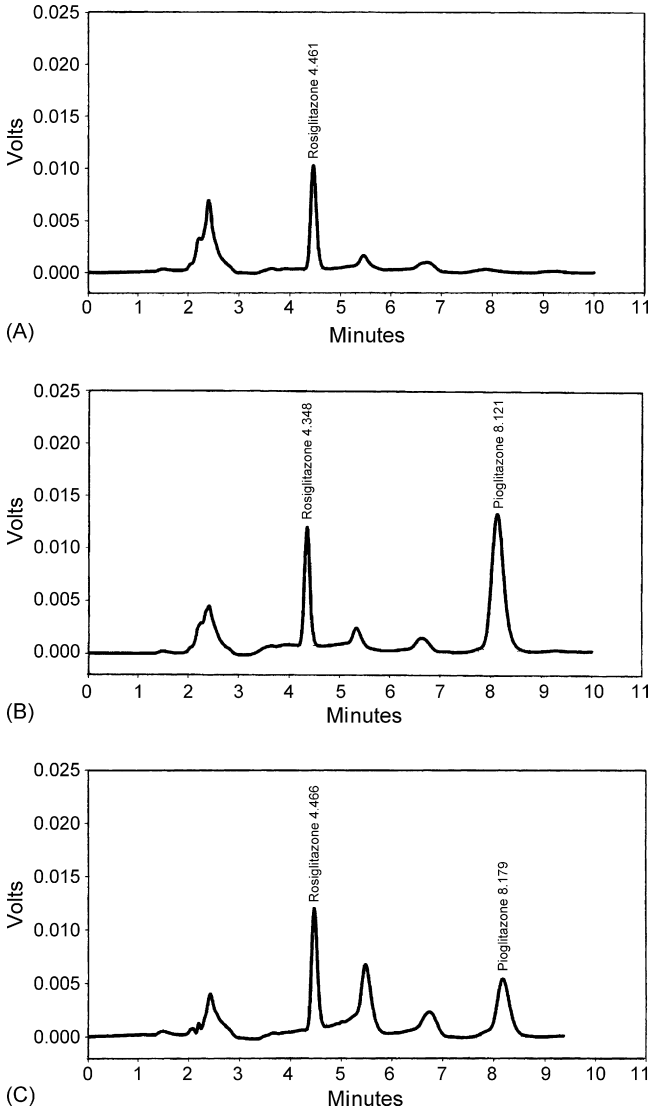

Fig. 3. Representative HPLC chromatograms of plasma taken from healthy Thai male volunteers at 0 h (A; LLOQ), 1.5 h (B; 1098.91 ng/ml) and 8 h (C; 586.11 ng/ml) after oral administration of a pioglitazone tablet.

concentration versus time profile is presented in Fig. 4. The mean values of pharmacokinetic parameters for the volunteers are shown in Table 6. The plasma levels of pioglitazone reached a maximum at 1.5 h after the administration (T_{max}) and thereafter the level declined with an elimination half-life ($T_{1/2}$) of approximately 5.2 h. T_{max} was in good agreement with the values reported by Eckland and Danhof [10] and by Hanefeld [11]. $T_{1/2}$ for the volunteers did not agree with other pharmacoki-

Table 6
Pharmacokinetic parameters of pioglitazone in 24 healthy Thai male volunteers after oral administration of a 30 mg pioglitazone tablet

Parameters	Mean ± S.D.
C_{max} (ng/ml)	1598.98 ± 659.04
T_{max} (h)	1.5 ± 0.8
AUC_t (μg h ml^{-1})	11.86 ± 5.04
AUC_∞ (μg h ml^{-1})	12.55 ± 5.13
$T_{1/2}$ (h)	5.2 ± 1.3

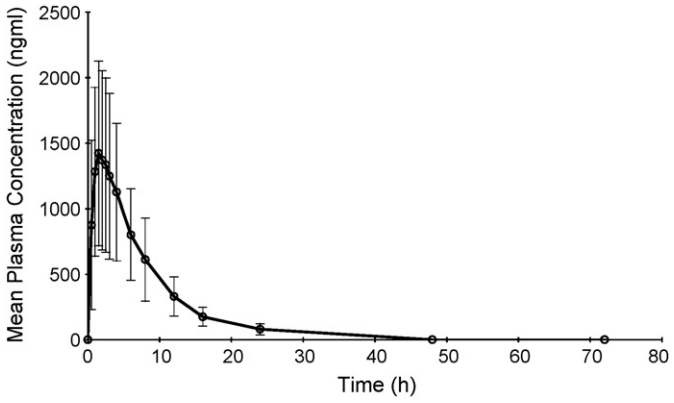

Fig. 4. Mean plasma concentration–time profile after oral administration of a 30 mg pioglitazone tablet to 24 healthy Thai male volunteers. Each point represents the mean ± S.D. ($n = 24$).

netic studies [10–12], whereas it was within the range of the values reported in the literature [3]. The maximum concentration (C_{max}) and the area under the curve (AUC) of pioglitazone obtained from this study were higher than the values reported by Wong et al. from an equivalent study design [13], which is due, at least in part, to the many factors affecting different ethnic groups. Moreover, those differences may depend on the difference between the analytical methods used, because the LC–MS/MS method used by Wong et al. [13] is more sensitive than the HPLC-UV method and should give more accurate data.

4. Conclusion

An HPLC method using a UV detector and an SPE cartridge proved to be simple and accurate for the determination of pioglitazone in human plasma. 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. Therefore, the HPLC method described here can be used for pharmacokinetic study of pioglitazone.

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