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# Sensitive and selective liquid chromatography–mass spectrometry method for the quantification of rosiglitazone in human plasma

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**Abstract**

A sensitive and selective high-performance liquid chromatography–electrospray ionisation–tandem mass spectrometry (HPLC–ESI–MS–MS) method for determination of rosiglitazone in human plasma has been developed. After the addition of the internal standard, plasma samples were precipited by acetonitrile. The compounds were separated on a proC18 column using a mixture of ammonium acetate buffer (0.02 M, pH 6.5) and acetonitrile (in the ratio of 47:53, v/v) as mobile phase. A Finnigan LCQdeca plus ion trap mass spectrometer connected to a Finnigan Surveyor HPLC was used to develop and validate the method. Linearity was established for the range of concentrations 1–1000 ng/ml with a coefficient of determination ($r^2$) of 0.999. The intra-day accuracy for rosiglitazone ranged from 110.0 to 99.2% at low, medium and high levels. The inter-day accuracy was less than 15%. The lower limit of quantitation (LLOQ) was identified reproducible at 1.0 ng/ml with a precision of 5.7%. After validation, the method was used to study the pharmacokinetic profile of rosiglitazone in five healthy volunteers after administration of a single oral dose (4.0 mg). The proposed method enabled the unambiguous evaluation and quantitation of rosiglitazone for pharmacokinetic, bioavailability or drug–drug interaction studies. A possible chromatography peak (*m/z* 121, its parent ion *m/z* 344) of *N*-demethyl rosiglitazone was observed at 3.49 min during determining rosiglitazone. This may be also a potential method for simultaneous determination of rosiglitazone and its metabolite *N*-demethyl rosiglitazone concentrations in plasma.


*Keywords:* Rosiglitazone; Human plasma; HPLC–ESI–MS–MS

## 1. Introduction

Rosiglitazone (**I**, BRL-49653), an oral antidiabetic agent of the thiazolidinedione class, has received regulatory approval for the treatment of type 2 diabetes as both monotherapy and the therapy in combination with other oral antidiabetic agents for its advantages of the therapeutic profile [1–3]. It is highly bound to plasma proteins (99.8%) and is primarily eliminated via metabolism in the liver by cytochrome P450 isoenzyme 2C8 [2]. The two major metabolites of rosiglitazone isolated via microsomal and animal studies are *para*-hydroxy rosiglitazone and *N*-demethyl rosiglitazone (Fig. 1). Rosiglitazone undergoes extensive metabolism and no unchanged parent drug is excreted in the urine. However, it was also found that the most common serious adverse effect of rosiglitazone had been pulmonary and peripheral oedema [4]. These adverse effects seem to be dose-(concentration) dependent and develop within the first few months of drug initiation or dose increment. Thus, availability of a simple and sensitivity method for determination of rosiglitazone in human plasma would aid in the evaluation of its bioavailability, or in the prediction of drug interactions when CYP2C8 inhibitors or CYP2C8 substrates concomitantly use with rosiglitazone.

Several methods had been published, but each had drawbacks limiting ease of use. Kolte et al. utilizes HPLC linked to ultraviolet detection or fluorescence detection to determine rosiglitazone in biological samples [5–10]. However, the methods required a large sample volume (1 ml) or at least 15 min to analyze a sample. Kim and Park developed a simplified HPLC fluorescence method to quantify rosiglitazone in human plasma [11]. But the

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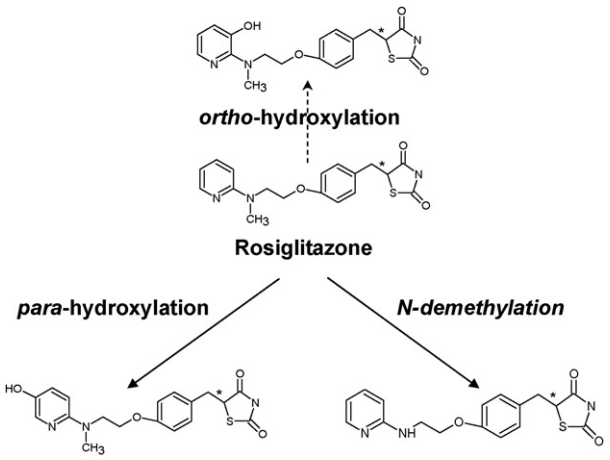


Fig. 1. Metabolic pathways of rosiglitazone *in vitro*. (*) Indicates the position of [$^{14}$C] radiolabe. Major pathways (solid arrow) and minor pathway (broken arrow).

lower limit of quantitation was only 10 ng/ml, which was not ideal for human pharmacokinetic studies of rosiglitazone if a single low dose of rosiglitazone was administered. In the published literatures, only one HPLC method with tandem mass spectrometric detection employed to quantify plasma rosiglitazone has been described, where Ho et al. used an API source in the positive ion mode [12]. The drug was isolated from equine plasma and urine by liquid–liquid extraction with 1,2-dichloroethane at acidic conditions before injected on HPLC–MS. The LLOQ was reported to be 10 ng/ml.

This paper describes a sensitive and selective high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS–MS) method with simplified sample processing to quantify rosiglitazone in human plasma. Applications of this method to the analysis of clinical samples from healthy volunteers suggested that it may be also a good method for the simultaneous quantitation of both rosiglitazone and its metabolites in human plasma.

## 2. Experimental

### 2.1. Chemicals and reagents

Rosiglitazone {5-(4-[2-(*N*-methyl-*N*-(2-pyridyl)amino)ethoxy]benzyl)–thiazolidine-2,4-dione} (**I**, Fig. 1), reference standard (purity, 99.8%) was purchased from Comen Chemical Company (Beijing, China). Citalopram (purity, 99.7%)) was gifted from Xian-Janssen Pharmaceutical Ltd., XiAn, China. Acetonitrile (HPLC grade reagents) was obtained from Tedia Company Inc.(Fairfield, America). Ammonium acetate (AR grade reagent) was obtained from Chemical Reagent Factory of Hunan (Changsha, Hunan, China). Blank human plasma was purchased from the Central Blood Bank of Xiangya hospital (Changsha, Hunan, China). Double distilled water was used throughout the procedure.

### 2.2. Standard solution and quality controls preparation

Rosiglitazone stock solution was prepared at a concentration of 1 mg/ml in acetonitrile in a 10 ml volumetric flask and stored at −20 °C. A 5.0 mg of citalopram was dissolved in 50 ml acetonitrile to prepare a 0.1 mg/ml stock solution. This was further diluted to yield 70 ng/ml citalopram as internal standard (IS). The rosiglitazone stock solution was further diluted in acetonitrile to give appropriate working solutions used to prepare the calibration solutions. All standard solutions were found to be stable at −20 °C for at least 2 months and at ambient temperature for 2 weeks when stored protected from light.

Quality control (QC) samples were prepared from a pool of blank human plasma spiked with three different amounts of rosiglitazone corresponding to the low, medium and high concentrations given in Table 1. Plasma aliquots were stored at −20 °C until assayed.

### 2.3. Sample preparation

All QCs, calibration curve and plasma samples of administered drugs were prepared as following processing. A 200 μl IS (70 ng/ml) and 200 μl acetonitrile was added to 200 μl plasma sample in microcentrifuge tubes and vortexed for 3 min, and centrifuged at 13,000 rpm for 10 min. The 500 μl organic layer was then removed and evaporated to dryness using Centrifugal concentrator in a heating block set at 45 °C. Dried samples were reconstituted with 80 μl of mobile phase and vortexed for 1 min. The upper organic layer was transferred to autosampler vials. A 10 μl aliquot was injected into the system.

### 2.4. HPLC conditions

Chromatography for separation and determination of the drug was carried out by applying the samples to a prepacked 5 μm (150 mm × 2.1 mm, i.d.) YMC-Pack ProC18 column (YMC Co; Ltd., Japan), using a Finnigan Surveyor high-performance liquid chromatograph (Thermo Finnigan, San Jośe, CA, USA). The combination of the mobile phase, prepared by mixing ammonium acetate buffer (0.02 M, pH 6.5): acetonitrile in the ratio of 47:53(v/v), and a flow rate of 0.20 ml/min was found to be adequate for the samples analysis. Separations were performed at room temperature.

### 2.5. MS–MS conditions

A ThermoFinnigan LCQ$^{deca}$ plus ion trap mass spectrometer (Thermo Finnigan) was used for all analyses. The instrument was operated in positive ESI ionization mode and was coupled to Surveyor HPLC with autosampler (Thermo Finnigan). Drug monitoring and quantitation were controlled by XCALIBUR. Operating conditions for the ESI source used in the positive ionization mode were optimized by constantly adding rosiglitazone standard solution (100 μg/ml) to the HPLC flow by a syringe pump via a T-connector in the infusion mode. The signal was optimized on the total ion current in MS mode, producing a transfer capillary temperature of 275 °C, a spray voltage of

Table 1
Precision (relative standard deviation, R.S.D.) and accuracy for low, medium and high concentrations of rosiglitazone in human plasma

| Rosiglitazone concentrations in human plasma (ng/ml) | Intra-day($n=3$) | | | Inter-day(3 days, $n=3$ each) | | |
|---|---|---|---|---|---|---|
| | Measured concentration (mean ± S.D., ng/ml) | R.S.D. (%) | Accuracy (%) | Measured concentration (mean ± S.D., ng/ml) | R.S.D. (%) | Accuracy (%) |
| 1 | 1.1 ± 0.1 | 9.1 | 110 | 1.1 ± 0.1 | 6.7 | 110 |
| 400 | 396 ± 17 | 4.3 | 99.0 | 396 ± 11 | 2.8 | 99.1 |
| 1000 | 992 ± 20 | 2.0 | 99.2 | 992 ± 14 | 1.4 | 99.2 |

5 kV, and a sheath gas flow of 30 units (units refer to arbitrary values set by the LCQ software). The above pretreated plasma samples containing rosiglitazone and its metabolites were added constantly to the HPLC flow by a syringe pump. On fragmentation, Rosiglitazone, *N*-demethyl rosiglitazone and IS produced specific daughter ions which were used for their specific detection using MS–MS–SRM mode. At the same time, the selection of ions and the collision voltages were optimized using LCQ software.

### 2.6. Assay validation

#### 2.6.1. Specificity and linearity

To test the specificity, six blank samples were tested for interference using the proposed extraction procedure and chromatographic–MS conditions, then these data compared with those obtained with an aqueous solution of the analyte at a concentration near to the lower limit of quantitation (1 ng/ml).

Calibration curves consisted of 11 standard concentrations of rosiglitazone in human plasma: 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 200.0, 400.0, 800.0 and 1000.0 ng/ml. Duplicate calibration curves were analyzed daily for 3 days. For each curve, the rosiglitazone peak area to IS peak area ratio was calculated and plotted against nominal rosiglitazone concentrations. Calibration curves for rosiglitazone were constructed by weighted ($1/y^2$) linear regression analysis.

#### 2.6.2. Precision and accuracy

Precision and accuracy were carried out using three different concentrations of QC samples on the same day and over 3 different days (Table 1). The concentrations of the analytes were determined from the calibration curve. Mean value, standard deviation, and relative standard deviation (R.S.D.) were calculated from QC values and used in the estimation of intra- and inter-day precision. Accuracy (bias) is expressed as the percent difference between the calculated mean concentration relative to the nominal concentration.

#### 2.6.3. Recovery

The recovery of rosiglitazone was determined at 1.0, 400 and 1000 mg/l by comparing the peak area ratio of rosiglitazone peak area to IS peak area after extraction of human plasma standards with the peak area ratio of rosiglitazone peak area to IS peak area obtained from injection of the same amount of rosiglitazone actonitrile standards. The absolute percent recovery was determined.

#### 2.6.4. Stability

Batches of high, medium and low QCs were prepared and subjected to three freeze-thaw cycles (−20 °C to room temperature) prior to processing and analysis. After each freeze thaw cycle, aliquots were extracted and analyzed. To determine stability of processed samples, three different concentration QC samples were prepared and subjected to repeated analysis over a 24 h period post-extraction.

### 2.7. Clinical application

Five male healthy volunteers (age range 19–23 years; weight range 62–72 kg) participated in the rosiglitazone pharmacokinetics study. The protocol was approved by Xiangya institutional Human Subjects Committee before study initiation, and individuals gave written informed consent before study enrollment. Rosiglitazone 4 mg (Avandia®, GlaxoSmithKline, Philadelphia, PA, USA) was administered orally with 200 ml water after an overnight fast. Blood samples for pharmacokinetic analysis of rosiglitazone were collected into tubes containing dilute heparin solution before and at 0.5, l, 2, 3, 4, 6, 8, 10, 12, 24, and 48 h after administration. Plasma was stored at −20 °C after collecting and until analyzed. A 200 μl IS (70 ng/ml) and 200 μl acetonitrile were added to 200 μl plasma samples using the proposed extraction procedure. A volume of 10 μl was automatically injected and analyzed according to the developed LC–MS–MS method. Plasma concentration–time curves were evaluated by one-compartmental analysis using Winolin 4.1.The maximum observed rosiglitazone concentration ($C_{max}$) and the time at which $C_{max}$ was observed ($T_{max}$) were reported.

## 3. Results

### 3.1. HPLC–MS–MS analysis

In the MS–MS experiments, the protonated precursor molecular ions $[M+H]^+$ of rosiglitazone (*m*/*z* 358), and the IS (*m*/*z* 325) were selected and fragmented by nitrogen gas collision in the ion trap at a relative collision energy of 38%. The mass spectra resulting from these fragmentations was acquired in the SRM mode at *m*/*z* 135 for rosiglitazone and *m*/*z* 262 for IS. Accordingly, the protonated precursor molecular ion $[M+H]^+$ of *N*-demethyl rosiglitazone(*m*/*z* 344) was selected and fragmentation was acquired in the SRM mode at *m*/*z* 121 at a relative collision energy of 35%. These product ions,

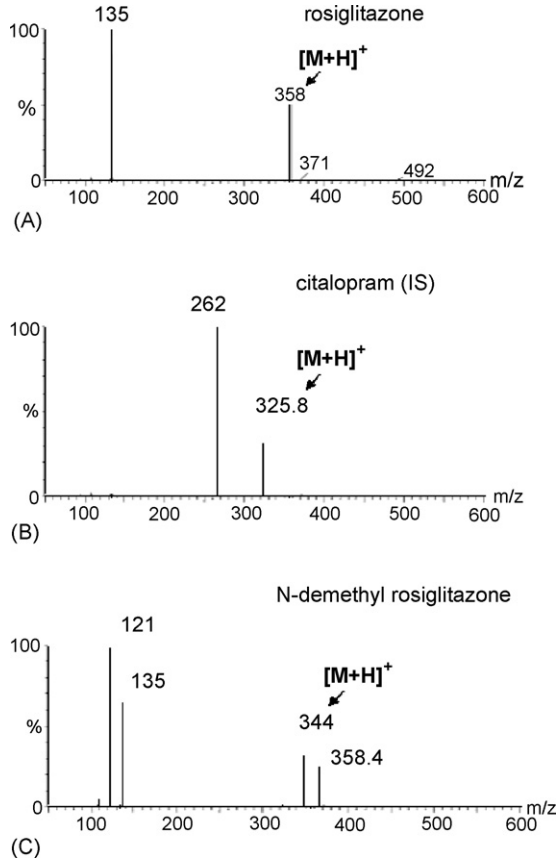


Fig. 2. Positive ion electrospray mass spectrum and product ion mass spectrum used in SRM for rosiglitazone (A), citalopram (B) and *N*-demethyl rosiglitazone (C) determination.

*m/z* 135 for rosiglitazone, *m/z* 262 for the IS and *m/z* 121 for *N*-demethyl rosiglitazone, were extracted for quantification (Fig. 2).

The total HPLC–MS–MS analysis time was 8 min per sample. The retention time of rosiglitazone and the IS were 5.76 and 3.24 min, respectively. The total ion current chromatogram of blank plasma samples indicated no endogenous peaks at the retention time ($t_R$) of rosiglitazone or internal standard. The product ion chromatograms of *N*-demethyl rosiglitazone (*m/z* 121) of prepared plasma samples obtained from healthy volunteers who had oral administered 4 mg rosiglitazone suggested a relationship of time–concentration curve. The corresponding SRM ion spectra of *N*-demethyl rosiglitazone in 4 h plasma of a volunteer was shown in Fig. 3. The retention time of *N*-demethyl rosiglitazone was 3.49 min.

### 3.2. Method validation

The specificity of the method was evaluated with regard to interference due to the presence of endogenous substances in the extracted human plasma. No significant interference at the retention time of the drug or internal standard was found.

Three separate rosiglitazone analytical series in duplicate were used to verify linearity of the calibration curve ($y = 3.653 \times 10^{-3} + 0.0121$) for the relevant range from 1 to 1000 ng/ml in human plasma. The correlation coefficients ($r^2$) were 0.999. The intra- and inter-day assay precision and accuracy for low, medium and high concentrations of rosiglitazone in human plasma were summarized in Table 1. The intra-day precision ranged from 2.0 to 9.1%, and accuracy (bias) was less than 15%. Inter-day precision and accuracy did not exceed 15%. The lowest standard concentration in the calibration curve was considered as the lower limit of quantitation. The lower limit of quantitation for rosiglitazone was proved to be 1.0 ng/ml, with mean CV (%) of 5.7% (Table 2). The accuracy of the technique was considered satisfactory, since between-day bias over the concentration range studied was found to be less than 15% and the LLOQ response was identifiable, discrete and reproducible with precision of lower than 20%.

Table 2
Back-calculated concentrations and statistics for precision and accuracy of rosiglitazone calibration standards of the lower limit of quantity (1 ng/ml)

| Theoretical concentration (ng/ml) | Calculated concentration (ng/ml) | | | | | Mean CV (%) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| 1 | 1.08 | 1.10 | 0.98 | 1.03 | 1.12 | 5.7 |

The data that represented the stability of rosiglitazone plasma samples at three QC levels over three cycles of freeze and thawing indicated that rosiglitazone was stable in human plasma for three cycles of freeze and thaw, when stored at −20 °C and thawed to room temperature. The post-preparative stability of QC samples kept in the autosampler which was set 4 °C for 24 h was also assessed. The mean recoveries of the low, mid and high QC levels were 97.7, 97.8 and 95.1%, respectively. The results suggested that rosiglitazone and internal standard could remain at 4 °C for at least 24 h, so we should handle samples within this period of time.

The samples were spiked with IS and analyzed as mentioned above. The absolute recoveries of rosiglitazone for three level QC calculated were 88.9, 93.2 and 97.6%, respectively. These results confirmed that the developed LC–MS–MS method was appropriate for the analysis of rosiglitazone.

### 3.3. Plasma sampling

The method was applied to analyze plasma samples obtained from healthy volunteers after the administration of a single dose of 4.0 mg rosiglitazone tablet. The mean plasma rosiglitazone concentration-time profile of five volunteers was represented in Fig. 4. The pharmacokinetic parameters were observed: $C_{max}$ was 516.7 ng/ml, which occurred at a $T_{max}$ of 3.8 h; the observed $t_{1/2}$ was 6.3 h.

## 4. Discussion

The HPLC–MS–MS system has become the instrument of choice for drug assay in biological fluids due to its inherent selectivity and sensitivity resulting in shorter chromatographic run time than the HPLC methods with other detectors. So we considered using HPLC–MS–MS for the detection of human

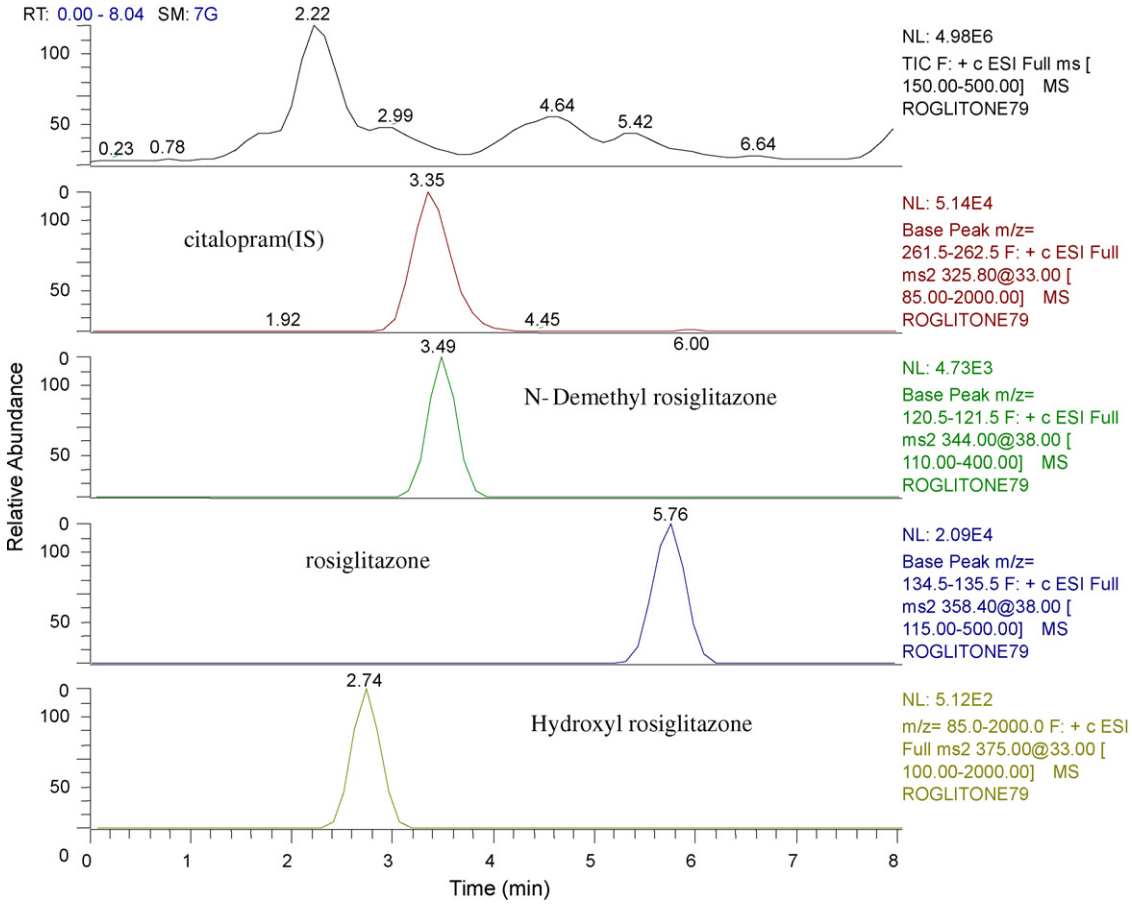


Fig. 3. SRM chromatogram of rosiglitazone, *N*-demethyl rosiglitazone and internal standard in human plasma sample 4 h after oral administration of 4 mg rosiglitazone.

plasma rosiglitazone concentration. In the present method, a small volume of human plasma was needed and the analysis time was also much shorter, 8 min versus 15 min [5]. The analysis time could be decreased even further by using a shorter column. This work proved a high selectivity and sensitivity for rosiglitazone determination in human plasma and analysis operated in MS–MS mode with an ESI interface. No endogenous interferences are in the regions where rosiglitazone, *N*-demethyl rosiglitazon and the IS eluted. The LLOQ of this approach was 1 ng/ml, thus it could meet the practical needs for 4 mg or lower oral dosage in humans while other methods were only reported to 8 mg or higher oral dosage. In order to evaluate a potential drug–drug protein binding interaction between glipizide and rosiglitazone, Lin et al. established a novel LC–MS–MS method for simultaneous determination of glipizide and rosiglitazone free fraction in human plasma using equilibrium dialysis for the separation of free (unbound) drug [13]. The sample preparation and drug extraction steps of this method were multiple and time-consuming. Although only unbound drug can play major role in pharmacodynamic interaction with receptors, the total drug measurement in plasma was regarded as classical approach in pharmacological studies. A new electrospray dual sprayer, LockSpray, was developed for cimetidine and rosiglitazone, on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (oa-Q-ToF). However, it was just suitable for accurate mass measurement for rosiglitazone, could not determine drug concentration in plasma [14]. Soglia et al. developed a micro-bore liquid chromatography–micro-electrospray ionisation–tandem mass spectrometry to predict the toxicity of rosiglitazone in vitro experiment. Rosiglitazone was detected as being conjugated by GSH following microsomal incubation [15]. This approach was always used in the drug discovery process and new drugs safety evaluation in preclinical stage. It was not suitable for drug concentration detection in plasma with some shortcomings, such as lower column peak capacity, which had the potential to result in poor resolution of peaks (conjugates) during analysis, and the narrow concentration range of standard curves was just from 5 to 300 nM.

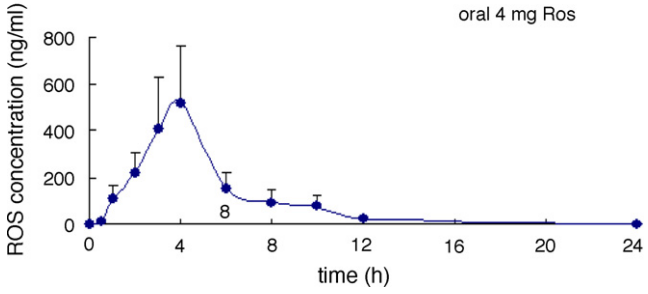


Fig. 4. Representative data showing mean plasma concentration–time profiles of five healthy volunteers after the administration of an oral single dose of 4 mg tablet of rosiglitazone. The error bars represented ± standard deviation.

It has become increasingly clear that most of adverse drug reactions or lack of clinical therapeutic effect occur because of the inhibition or induction of P450 enzymes. So it is very important to evaluate P450 enzymes activity and time-monitor plasma drug concentration in clinical management. Recent in vivo studies have demonstrated the importance of CYP2C8-mediated metabolism as a target of drug–drug interactions [16]. Walsky used *N*-desethylamodiaquine as CYP2C8 probe drug, and reported a validated sensitive, moderate-throughput high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) assay to it [15]. Since rosiglitazone was predominantly metabolized by CYP2C8, it may have potential use as an in vivo probe of this enzyme. Availability of a simple method for determination of rosiglitazone in human plasma would aid in the evaluation of rosiglitazone as a CYP2C8 probe.

In this paper, we introduce a more simplified, sensitive and selective HPLC–MS–MS method than previous reported method to quantify rosiglitazone in human plasma, or for bioavailability or bioequivalence studies. It is adapted for clinical quantification of rosiglitazone. A possible chromatography peak (*m*/*z* 121, its parent ion *m*/*z* 344) of *N*-desmethyl rosiglitazone was observed at 3.49 min during determining rosiglitazone process. Thus, it was also considered as a potential method for simultaneous determination of rosiglitazone and its metabolite *N*-desmethyl rosiglitazone concentrations in plasma.

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