Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1097 (2005) 74–83
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry for determination of trace rosiglitazone in urine

Chi-Chi Chou [a], Maw-Rong Lee [a,*], Fu-Chou Cheng [b], Dar-Yu Yang [c]

[a] *Department of Chemistry, National Chung-Hsing University, 250 Kuo-Kuang Road, Taichung 40227, Taiwan*
[b] *Department of Medical Research, Taichung Veterans General Hospital, Taichung 40705, Taiwan*
[c] *Department of Emergency, Taichung Veterans General Hospital, Taichung 40705, Taiwan*

Received 2 April 2005; received in revised form 25 July 2005; accepted 5 August 2005
Available online 6 September 2005

Abstract

This project evaluated solid-phase extraction (SPE) combined with liquid chromatography–tandem mass spectrometry (LC–MS/MS) to determine the trace amount of rosiglitazone in human urine. The analytical performance of four modes of LC–MS and tandem MS operation (atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), positive and negative ionization) was compared for two mass spectrometers, a triple-quadrupole and a quadrupole ion trap instrument. Rosiglitazone was extracted from urine using a SPE cartridge of 50 mg C8 sorbent and acetonitrile used as the eluting solvent. Samples were then separated on a RP18 column interfaced with a tandem mass spectrometer. The recovery of rosiglitazone was greater than 91.2%. The urine assay combining SPE and LC–APCI-MS/MS of triple-quadrupole was proved a very selective and sensitive method for determination of trace rosiglitazone. The assay was linear over a wide range, with a lower limit of quantification of 0.1 ng/mL using 1 mL of urine. The intra- and inter-day precisions were <9.8% and <7.9%, respectively, and the accuracies were in the range 91.0–103.6%. The rosiglitazone concentration profile in human urine was also determined. The results of this study reveal the adequacy of SPE–LC–APCI-MS/MS method for analyzing rosiglitazone from diabetic patients' urines. The concentrations of rosiglitazone were detected to range from 760 to 164 pg/mL.

Keywords: Rosiglitazone; Urine; Solid-phase extraction; Liquid chromatography; Tandem mass spectrometry

1. Introduction

Rosiglitazone, [(±)-5-[[4-[2-methyl-2-(pyridinylamino)ethoxy]phenyl]methyl]-2,4-thiazoli-dinedione-(*Z*)-2-butenedioate (1:1)] (Fig. 1A) is a potent synthetic peroxisome proliferators-activated receptor gamma (PPAR-γ) agonist that decreases hyperglycemia by reducing insulin resistance in patients with type 2 (noninsulin-dependent) diabetes as both monotherapy and in combination with oral antidiabetic agents [1,2]. Rosiglitazone is in a class of drugs called thiazolidinediones which work by increasing insulin sensitivity in target tissues, as well as decreasing hepatic gluconeogenesis [3,4]. Some concern about class safety has been raised by the removal of troglitazone from the market due to human hepatotoxicity and severe, irreversible liver failure [5]. Although rosiglitazone does not seem to share this problem, the mechanisms of troglitazone reactions are not clearly elucidated. However, there is a substantial amount of evidence that chemically reactive metabolites are involved for the liver toxicity [6]; the knowledge about metabolic steps is a prerequisite for toxicological risk assessment. As a result, methods for rapidly detecting and characterizing rosiglitazone and its metabolites are highly desired in this class of drugs. Rosiglitazone was derived from a metabolite of ciglitazone and found to be much more potent than other classes of thiazolidinediones such as pioglitazone, ciglitazone and englitazone [7]. This drug contains a thiazolidinedione core, but differs from other thiazolidinediones in the presence of an aminopyridyl side chain [8]. Such substitutions among side chains are believed to be responsible for differences in disposition, antidiabetic efficacy and metabolism among thiazolidinediones [9]. Rosiglitazone is primarily eliminated via metabolism in the liver by cytochrome P450 isoenzyme 2C8. Following oral treatment rosiglitazone is rapidly absorbed (T_{max} 1–2 h) with

* Corresponding author. Tel.: +886 4 2285 1716; fax: +886 4 2286 2547.
E-mail address: mrlee@mail.nchu.edu.tw (M.-R. Lee).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.08.033

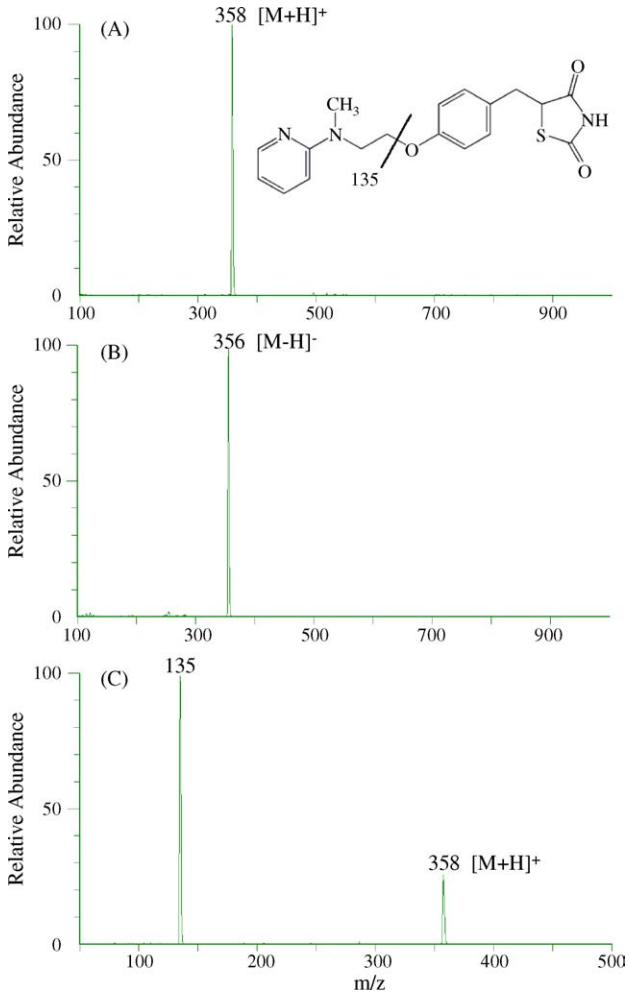

Fig. 1. APCI mass spectra of rosiglitazone: (A) positive ion full-scan mass spectrum, (B) negative ion full-scan mass spectrum and (C) positive ion product ion spectrum of *m/z* 358.

an elimination half-life of approximately 3–5 h in plasma [10–12]. The major routes of metabolism in humans were *N*-demethylation and hydroxylation with subsequent conjugation [12].

Literature survey reveals that few liquid chromatography (LC) methods have been reported for the determination of rosiglitazone in bulk drug or human plasma. The LC techniques used were including LC with ultraviolet (UV) detection [13–16] and with fluorescence detection [17–21]. Nowadays liquid chromatography coupled with mass spectrometry (LC–MS) has gained in popularity and been successfully applied to a wide variety of drugs in biological matrices. LC–MS, unlike GC–MS, is not limited by such factors as nonvolatility and high molecular weight, and enables the determination of both lipophilic parent drugs and hydrophilic metabolites without the need for derivatization or hydrolysis. The relatively recent introduction of tandem mass spectrometry coupled to liquid chromatography (LC–MS/MS) has largely improved the performance of the technique by enhancing sensitivity and analyte identification; therefore, it has led to increasing usage in high-throughput analysis and metabolic studies. Several of LC–MS/MS applications for analysis of rosiglitazone have been developed recently [22–24]. However, these works only focus on quantitative determination of rosiglitazone in pharmaceutical or bio-assay plasma samples, but none could be found that focus on the analysis of rosiglitazone in human urine. Usually, urine samples are more readily available for drug analyzing in pharmacokinetic and bioavailability studies. In addition, urinary excretion is an essential issue in determination of renal clearance of the drugs investigated. From previous reports, rosiglitazone excreted from urine is only small amount and unusually found in the urine of patients receiving therapeutic doses of this anti-diabetic. For the low parent drug levels in urine, analytical methods for the detection of rosiglitazone must be specific and sensitive in order to be able to adequately measure this drug at low levels. However, to our knowledge, there is no method available to analyze this drug at the sub-ppb levels. The enrichment and sample cleanup steps are indispensable for the low concentrations occurring in the urine. A sample preparation step is often necessary to isolate the compound of interest from the sample matrix, as well as to concentrate and purify the analytes. For biological matrices, selective analysis for drugs from a variety of matrices is often performed today by solid-phase extraction (SPE) [25]. SPE is a robust method, which offers the advantages over conventional liquid–liquid extraction (LLE) techniques by having a generally higher drug recovery, lower consumption of organic solvent and the speed of the extraction process, mainly for laboratories providing large number of analyses with rapid reporting time. Using SPE with mass spectrometry, being a more efficient method than both UV and fluorescence detection, may overcome false positives problems result from basal compounds of matrices or concomitant therapies. Müeller et al. [26] and Briem et al. [27] investigated the effects of SPE and protein precipitation on ion suppression during subsequent electrospray ionization MS. Both groups found that SPE had a greater impact on reducing ion suppression. Consequently, SPE is often used for desalting and preconcentration of samples prepared for GC–MS or LC–MS analysis.

In this study, a simple and sensitive method for the determination of rosiglitazone in human urine was developed by using SPE combined with LC–MS/MS. The SPE procedure has been optimized in order to obtain sufficiently high recoveries for rosiglitazone, and the LC and MS/MS conditions were also investigated in order to detecting a very low concentrations for this drug. The analytical performance of the atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) techniques, both in the positive ionization (PI) and negative ionization (NI) mode was compared. This was done for two different mass spectrometers, a triple-quadrupole (TQ) and quadrupole ion trap (QIT) instrument, since their differences in design may affect the MS mode selection. The feasibility of applying the method to determine rosiglitazone in real samples was examined by profiling this drug from human urine after an oral administration of a single dose of Avandia® and analyzing urine samples from rosiglitazone dosed patients.

2. Experimental

2.1. Chemicals and reagents

Rosiglitazone maleate standard (74.3% of rosiglitazone free base) and Avandia® tablets (rosiglitazone; 4 mg) were kindly supplied by GlaxoSmithKline (SB Pharmaco Puerto Rico Inc. Cidra, Puerto Rico). HPLC-grade of acetonitrile, methanol and ethanol were purchased from Merck (Darmstadt, Germany). Analytical grade of formic acid, ammonium formate, acetic acid, ammonium acetate and glycine were also obtained from Merck. Sodium hydroxide (99%) and sodium chloride (99.8%) were purchased from Riedel-de Haën (Seelze, Germany). Hydrochloric acid (36.5%) was from Fisher Scientific (Hampton, NH, USA). A stock solution of rosiglitazone at 1.0 mg/mL was prepared by dissolving the solid bulk drug in acetonitrile and stored at −30 °C. This solution was further diluted to yield the appropriate working solutions with acetonitrile. One set of analyte working solutions was used to prepare calibration standards, and the other set was used to make quality control (QC) samples. All concentrations are expressed in terms of pure base. For comparison of TQ and QIT mass analyzers with various ionization modes, calibration curves concentrations of 0.1–2000 ng/mL were prepared by serial dilution of stock solution with acetonitrile. Pooled drug-free human urines were obtained from healthy volunteers, stored at −30 °C and allowed to thaw at ambient temperature prior to use. Urine samples were prepared by spiking with standard rosiglitazone solution. The urine samples collected of rosiglitazone dosed patients were kept frozen at −30 °C until further treatment. All solvents used in this study were of HPLC grade or analytical-reagent grade. The laboratory purified water (>18 MΩ) was obtained from a SG-Ultra Clear water purification system (SG Water, France).

2.2. Solid-phase extraction device

Solid-phase extraction was used to preconcentrate the analyte from urine. Optimization of the SPE was then studied with respect to extraction efficiency for rosiglitazone used to spike a 1 mL urine sample. The 12-fold Visiprep SPE vacuum manifold was purchased from Supelco (Bellefonte, PA, USA). The sorbents including C8, C18 and SCX were obtained from Chrom Expert Company (Sacramento, CA, USA). The laboratory-packed cartridges were made using 10–200 mg of sorbent packed into 3 mL polypropylene syringes, retained by two polyethylene frits (20 μm pore size). Cartridges were conditioned with, successively, 3 mL of methanol, 3 mL of water and 1 mL of 10 mM ammonium acetate buffer (pH 8.0). All conditioning steps used a flow rate of 3 mL/min. The 1 mL urine samples were then extracted by vacuum aspiration through the SPE cartridges at a flow-rate of 1 mL/min. After the sample loading, interferences were washed off the cartridges with 1 mL of water at 1 mL/min. Analytes were then eluted from the SPE cartridges using 3 mL of acetonitrile. Elution flow was set at 1 mL/min. The eluates were evaporated to dryness at 45 °C, under a gentle stream of nitrogen gas. The dry residues were reconstituted in 500 μL of acetonitrile and vortex mixed for 15 s. A 5 μL portion of the resulting solutions were injected into the LC–MS/MS system. To maximize the precision of the SPE analysis, the flow rate was required to control carefully in sample extraction and elution steps.

2.3. Chromatographic conditions

Analyses were carried out on two LC systems under identical chromatographic conditions. One was a Dynamax ProStar 210 liquid chromatograph system (Thermo Finnigan, San Jose, CA, USA), consisting of two LC pumps, and the other was a Surveyor liquid chromatograph system (Thermo Finnigan, San Jose, CA, USA). The rosiglitazone was separated from the rest of the urine extract compounds on a Waters XTerra RP18 (25 cm × 4.6 mm, 5 μm) LC column (Waters Corp., Milford, MA, USA), which was preceded in-line by a precolumn filter of 3 mm frit (Supelco, Bellefonte, PA, USA). The mobile phase was filtered using a filter (0.45 μm), and degassed by using vacuum followed by sonication. The LC operating conditions were isocratic separation using a mixture of acetonitrile–10 mM ammonium formate of the buffer adjusted with formate acid (pH 5.0) (72:28, v/v) eluent at a 0.5 mL/min flow-rate and ambient temperature (25 ± 2 °C). The injection volume was 5 μL and the entire column effluent was directed into the mass spectrometer.

2.4. Triple-quadrupole mass spectrometer conditions

A Varian 1200L triple-quadrupole LC–MS (Varian, Walnut Creek, CA, USA), equipped with an ESI source and an APCI source was used for the MS/MS analyses coupled to the Dynamax ProStar 210 LC. The mass spectrometer was operated at low resolution for both Q1 and Q3 in selected reaction monitoring (SRM) mode. The data acquisition software Varian MS workstation, version 6.2 was used for instrument control, data acquisition and data handling. Analysis was performed in both positive ionization (PI) and negative ionization (NI) modes. Voltages across the capillary, lens and the quadrupoles were tuned by an automated procedure to maximize the signal for the ion of interesting. The parameters of the ionization efficiency were optimized by evaluating the sensitivity using flow injection analysis (FIA) with the mobile phase at 0.5 mL/min. Conditions of all the instrumental parameters were optimized with regard to maximum signal intensity of the protonated molecular ion $[M+H]^+$ by injections of 5 μL standard solution (100 ng/mL) through a sample loop. MS/MS performed was based on collision-induced dissociation (CID) of specific precursor ion and the generation of the characteristic fragment ions. Q1 was scanned over a range of m/z 100–1000 for precursor ion optimization. The protonated molecular ion, $[M+H]^+$ of the analyte was used as the precursor ion for subsequent MS/MS experiments. Subsequently, product ion spectra were obtained by scanning Q3 over a mass range of m/z 50–500. Both Q1 and Q3 quadrupoles were set at unit resolution. The effects of different collisional gases on MS/MS efficiencies including nitrogen, helium and argon were investigated. For each of the three colli-

sional gases, the collisional energy and pressure optimized for the compound selected was used. During SRM analysis mode, mass peak width was 1.0 mass unit at half height for both Q1 and Q3, the scan time was 1 s and 5 μscans were averaged for each spectrum.

2.5. Quadrupole ion trap mass spectrometer conditions

The LC–QIT/MS system consisted of a Surveyor LC system and a LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source and an APCI source, and operated in both positive and negative polarity. The data acquisition software used was Xcalibur (ThermoFinnigan), version 1.2. The instrumental parameters of mass spectrometer were all optimized with regard to maximum signal intensity of $[M+H]^+$ ions by using FIA and injections of 5 μL standard solution (100 ng/mL) in the same way for the TQ with the mobile phase at 0.5 mL/min. MS/MS was performed based on CID of specific precursor ion by collisioning with the helium damping gas (99.9995% purity) present in the trap for 30 ms, and the isolation width fixed at 2.0 mass unit. The product ion spectra were obtained by scanning a mass range of *m*/*z* 50–500. During SRM analysis mode, the injection time was 600 ms, the scan rate was 180 μs/u and 3 μscans were averaged for each spectrum.

2.6. Method validation

The method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and recovery. Urine calibration standards were prepared daily by adding 25 μL of the appropriately diluted standard into 1 mL of drug-free urine to provide final concentrations of rosiglitazone in urine of 0.05, 1, 10, 50, 100, 200 and 500 ng/mL. Three replicate analyses were performed for each concentration to evaluate linearity. The peak areas were used for quantification through the calibration curves of rosiglitazone. LOD and LOQ were defined as concentrations in a urine sample resulting in peak areas with signal-to-noise ratios (S/N) of 3 and 10, respectively. The QC samples (0.1, 1, 10, 50 and 100 ng/mL) were prepared by adding 50 μL of appropriate rosiglitazone standard solution to 10 mL of drug-free urine. Accuracy and precision of the assay were determined by generating intra- and inter-day variability data from a series of QC samples in the range of 0.1–100 ng/mL, either extracted five times in a single day or extracted three times in three different days, respectively. Both intra- and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis. Accuracy was reported as percent accuracy and expressed as the ratio of mean measured concentration to spiked concentration. To verify the specificity, control samples containing no added analyte were prepared by using drug-free urine samples from five different donors. The absence of endogenous compounds at the retention time of the analyte was investigated. The analytical recovery of rosiglitazone for urine samples was determined by analyzing triplicates of QC samples at concentrations of 0.1–100 ng/mL.

2.7. Administration samples

The developed SPE–LC–MS/MS procedure was used to investigate the urine profiles of rosiglitazone after a single oral dosed. The healthy female volunteer received a single dose of 4 mg of rosiglitazone (Avandia® F.C. tablet 4 mg). Seventeen urine samples were collected before (0 h) and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 16 and 24 h post-dosing. Urine samples were stored at −30 °C until analysis.

3. Results and discussion

3.1. Optimization of MS conditions

The ESI and APCI modes were used with both PI and NI modes for TQ and QIT mass analyzers to evaluate the most sensitive ionization method for the analyte. The concentration of 100 ng/mL rosiglitazone was used in this study with FIA. In APCI experiments, protonated molecular ion, $[M+H]^+$, *m*/*z* 358 was obtained in PI mode as the base ion, and less fragment ions were observed for both analyzers (Fig. 1). The ESI mass spectrum of rosiglitazone obtained the similar result. There are two nitrogen atoms at *N*,*N*-dimethylpyridin-2-amine moiety of rosiglitazone as proton acceptors. The π-orbital of pyridine resonances stabilization with the lone paired electrons on the nitrogen of trimethylamine moiety; therefore, the nitrogen of pyridine is easy to protonated. In the NI APCI mode experiments, the peak due to the deprotonated molecule $[M-H]^-$, *m*/*z* 356 was obtained as the base ion (Fig. 1B). There is due to the nitrogen of thiazolidinedione moiety of rosiglitazone as proton donor. The 2,4-dione double bonds can be resonated with the lone paired electrons on nitrogen of thiazolidinedione let to deprotonate on nitrogen under the critical conditions. The deprotonated efficiency increased with increasing the basicity of mobile phase or temperature of ionization. Our preliminary experiments indicated that, the signal of $[M-H]^-$ increased significantly when the temperature of vaporizer increased from 300 to 500 °C and to reach maximum at 525 °C. Besides, the intensity of $[M-H]^-$ ion was not observed while the vaporizer temperature below 300 °C. In the NI ESI mode, rosiglitazone seemed to be very poorly ionized without heating, even increasing the basicity of mobile phase. The similar results were obtained on the two different mass spectrometers; therefore, the NI ESI mode was not considered further for using to analyze rosiglitazone. The optimum MS parameters of both analyzers are indicated in Table 1.

3.2. Optimization of MS/MS conditions

In MS/MS studies, both ESI and APCI interface were used in conjunction with the FIA for optimization of the MS/MS parameters. The product ion mass spectra of protonated molecule ion, $[M+H]^+$, *m*/*z* 358 of rosiglitazone were obtained in the scan range of *m*/*z* 50–500. As can be seen from Fig. 1C, the major ion at *m*/*z* 135 corresponds to the loss of 5-(4-hydroxybenzyl)-thiazolidine-2,4-dione (223 u) from the protonated molecule ion of rosiglitazone. Therefore, in this assay, the mass transition pat-

Table 1
Optimal instrument parameters for rosiglitazone

Condition	ESI[a] (ion trap)	APCI[a] (ion trap)	ESI[a] (triple-Q)	APCI[a] (triple-Q)	APCI[b] (triple-Q)
Nebulizing gas flow rate (L/min)	1.05	0.45	1.25	0.75	0.63
Drying gas flow rate (L/min)			4.2	1.6	0.8
Auxiliary gas flow rate (L/min)	7.5	0		0.6	0.9
Corona current (μA)		7		8	−8
Spray voltage (kV)	5		2		
Capillary voltage (V)	90	90	75	75	−75
Vaporizer temperature (°C)		350		500	525
Capillary temperature (°C)	250	200	300	50	200
Housing temperature (°C)			45	50	53
Shield voltage (V)			700	600	−400
Tube lens offset (V)	−25	−10			
Lens voltage (V)	−16	−40	−2	−2	1
Octapole 1 offset (V)	−2	−2			
Octapole 2 offset (V)	−5.5	−8			
Octapole RF amplitude (V)	600	400			
Quadrupole 0 offset (V)			−2.44	−2	4.5
Quadrupole 1 offset (V)			−0.5	−1.1	1.4
Quadrupole 1 guide (V)			−3	−6	5.4
Quadrupole 2 offset (V)			−10	−15	11.5
Quadrupole 3 offset (V)			−11	−10	13
Quadrupole 3 guide (V)			0	0	2

[a] Positive ion mode.
[b] Negative ion mode.

tern, *m/z* 358 → 135 was selected to monitor rosiglitazone in PI SRM analysis for both ESI and APCI modes. The extent of the fragmentation of the parent ion depends on the collisional energy and the collisional gas pressure in the collision cell of TQ MS. The effects of nitrogen, helium and argon gases on the fragmentation efficiencies were also investigated to generate the maximum MS/MS performance of TQ MS. For each of the three collisional gases, the collisional energies of 18–36 eV and pressure of 0.07–0.3 Pa were studied. Within the conditions tested, the maximum performance was reached with argon at the collisional energy of 24 eV and gas pressure of 0.1 Pa.

The product ion of negative ion APCI mass spectrum of the deprotonated ion $[M-H]^-$, *m/z* 356, of rosiglitazone was studied with different gases, including nitrogen, helium and argon. Whatever the kind of collisional gas used and no matter how less energy and pressure set, the deprotonated ion $[M-H]^-$ was fragmentized completely and none of any stable product ion was obtained. This is due to the thermal energy which from the vaporizer temperature induced the dissociation of parent ion. Hence, SRM scan mode of NI APCI was not considered in this study, and the selected ion monitoring (SIM) scan mode was used instead of.

For QIT MS, the extent of the fragmentation of the parent ion depends on the collisional energy and the collisional gas pressure in the ion trap. The pressure of helium collisional (damping) gas was limited by instrumental characteristics of the restrictions of QIT and fixed at about 0.1 Pa in this study. For the optimum MS/MS performance, the collision energy was set to be 1.6 V peak-to-peak of resonance excitation RF voltage to produce a nearly 100% fragmentation of the $[M+H]^+$, *m/z* 358 of rosiglitazone and obtain the maximum intensity of product ion at *m/z* 135.

3.3. LC optimization

Different mobile phase compositions were evaluated for chromatographic peak shape, speed and ionization efficiency. To see whether pH has an effect on elution and the MS signal intensity, the buffer pH was varied using formic acid, acetic acid, ammonium formate and ammonium acetate. The pH of the mobile phase was found to markedly affect the retention of rosiglitazone. At pH below 3.0, rosiglitazone was not retained and was subject to considerable matrix effects. An increase in pH from 3.0 to 5.0 caused the rosiglitazone retention time to shift between 5.8 and 11.6 min and below pH 5.0, band broadening and peak tailing were observed. The retention time decreased from 11.6 to 9.8 min by increasing the pH from 5.0 to 7.0. The pK_a value of rosiglitazone is between 6 and 7 in water. Because of the high-percentage (65%) acetonitrile solvent mixtures, the real pH of the system is slight increased. At pH 5.0, rosiglitazone shifts significantly to the neutral form, which has a higher affinity for the static phase, thereby increasing the retention time. Moreover, the amounts of rosiglitazone detected dropped about 60 and 99% at pH 6.0 and pH 7.0, respectively. We believe that this change in rosiglitazone ionization efficiency is related to the higher pH of the mobile phase, leading to deprotonated of rosiglitazone. For both ESI and APCI modes, acetonitrile–ammonium formate buffer was found to be the best eluent combination. The good results obtained with ammonium formate buffer may be explained by the stronger acidity of formic acid compared to acetic acid in the gas phase [28]. The higher anion concentration of the former acid at pH 5.0 will cause the ionization efficiency to be higher. With respect to the peak sharp in chromatographic resolution, the mobile phase composed of different acetonitrile–buffer mixtures was optimized

Table 2
Estimated limits of detection and linear range for different ionization modes and analyzers by LC–MS/MS methods

Ionization mode (analyzer)	Linear range (ng/mL, $n = 6$)	Correction coefficient (r^2)	LOD[c] (ng/mL)	LOQ[d] (ng/mL)
ESI (ion trap)[a]	25–2000	0.9997	2.2	7.3
APCI (ion trap)[a]	1–500	0.9996	0.9	3.2
ESI (triple-Q)[a]	0.5–1500	0.9997	0.3	0.9
APCI (triple-Q)[a]	0.1–2000	0.9995	0.1	0.3
APCI (triple-Q)[b]	1–1000	0.9995	0.2	0.7

[a] Positive ion–SRM mode (m/z 358 → 135).
[b] Negative ion–SIM mode (m/z 356).
[c] LOD = limit of detection, determined as S/N = 3.
[d] LOQ = limit of quantification, determined as S/N = 10.

in terms of both band broadening and ionization efficiency. In order to obtain maximum performance, we investigated the optimum acetonitrile percentage varied from 60 to 90%. In this study higher-percentage acetonitrile mixtures gave symmetrical peaks and enhanced detection sensitivity by facilitating desolvation of the mobile phase. The best efficiency corresponding to 72% acetonitrile was found. As a result of these experiments, the best compromise was to adjust the pH to 5.0 with 10 mM ammonium formate–formic acid and 72% acetonitrile where the analyte retention was adequate for high sample throughput and maximum signal intensity. Under these optimum conditions, rosiglitazone gave retention time of 7.8 min and eluted with no detectable interfering compounds. Reproducibility of retention times was checked by repeated injections of standard solutions. Retention times resulted in 0.5% relative standard deviation ($n > 50$), which presents a good value for qualitative and quantitative analysis.

3.4. Comparison of LC–MS/MS methods

To evaluate the optimum detection technique for trace analysis of rosiglitazone, performance of the methods with various ionization and polarity modes of both analyzers were compared with respect to the limits of detection (LODs), limits of quantification (LOQs) and linearity ranges obtained. The instrumental LODs and LOQs were defined as concentrations in a standard solution resulting in peak areas with signal-to-noise ratios (S/N) of 3 and 10, respectively. From the results in Table 2 indicate the squared correlation coefficients (r^2) were in the range of 0.9995–0.9997 depending on the ionization modes and analyzers. LODs obtained were ranged from 0.1 ng/mL for the PI APCI–SRM mode (TQ) to 2.2 ng/mL for the PI ESI–SRM mode (QIT). The APCI source offered better LODs than those obtained in ESI source for both analyzers. The mechanism of matrix effect in ESI is usually accepted that it results from the ionization competition between the different species eluted from the column [29,30], however, APCI is less susceptible to matrix effect than ESI source because ionization takes place in the gas phase [31,32]. TQ achieved lower LODs than that obtained in QIT, and its linearity range was larger with two orders (Table 2). This is due to the decrease of trapping efficiency of target ions in the present of systemic interferences can affect the LODs and linearity in the QIT instrument. Regarding the performance of MS and MS/MS, the NI APCI offers a good sensitivity in SIM detection mode; however, the MS/MS system provides the highest selectivity to get lower LOD. On the basis of MS experimental results, PI APCI was adopted for generation of the precursor protonated molecule $[M + H]^+$ in MS/MS experiments of this work. The TQ mass analyzer was chosen to achieve best sensitivity for rosiglitazone and lower levels of background noises.

3.5. Optimization of extraction parameters

For the optimum conditions of solid-phase extraction, a number of parameters were studied for spiked urine samples containing 10 ng/mL rosiglitazone. Since the extraction is a complex process and according to the character of the sample (polarity, solubility, pK_a, ...), the sorbent and elution solvent should be carefully chosen. The optimization of an appropriate SPE cartridge with different sorbent materials plays a key role in the achievement of high and reproducible recovery for analytes. To our knowledge known method using C18 sorbent for extraction of rosiglitazone was taken [12]. Because there was none result of earlier performed study on the suitability testing of SPE sorbents for rosiglitazone, apolar, cation-exchange alkyl-silica and mixed mode (reversed phase and cation-exchange) sorbents with using laboratory-packed cartridges including, C8, C18, SCX, three different mixed modes (4:1, 5:1, 6:1) of both C8/SCX and C18/SCX were examined. Fig. 2 shows the comparison extraction performance of rosiglitazone in urine with a various kind of sorbents under their individual optimum conditions. For rosiglitazone ion, there are two cation-exchange sites of the hydrogen ions on the nitrogen atoms of *N*,*N*-dimethylpyridin-2-amine moiety.

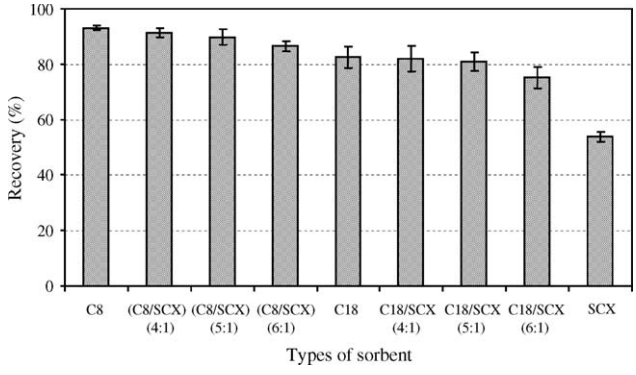

Fig. 2. The effect of various sorbents on the recovery of 10 ng/mL rosiglitazone in urine.

Rosiglitazone is extracted via the hydrophobic interaction of its aromatic moiety and the C8- or C18-chains and/or the electrostatic attraction between the sulfonic acid moiety and cation-exchange sites of rosiglitazone ions. However, ionic interaction is not as strong as hydrophobic interaction herein; therefore, for extracting rosiglitazone from urine samples, an apolar C8 sorbent was shown to present the most selective material for cleanup, and offered the best extraction efficiency with limited variation. This fact is related to the analytes polarity; C8 has a medium polarity between C18 and SCX, and its polarity is similar to the target analyte. As a consequence, the C8 sorbent was chosen for extraction rosiglitazone in this study.

The different amounts of C8 sorbent used to evaluate the extraction efficiency. All results shown in this article were obtained with a urine volume of 1 mL. The results showed that 50 mg C8 sorbent would be sufficient to retain analytes onto the sorbent completely for a 1 mL aliquot of urine sample. The high recovery of 50 mg of sorbent proves that the capacity of the SPE cartridge was not exceeded and no breakthrough occurred. Hence, 50 mg C8 sorbent was chosen for further study.

The extraction steps were properly optimized by changing the nature and the volume of the eluent as well as the composition and the volume of the conditioning and the washing solvent. Thus, conditioning step was tested with different solvent such as methanol, acetonitrile and water for solvation and aqueous buffers with variant pH for equilibrium of the sorbent with different volumes. However, best results were given when methanol, water and aqueous buffer were used at the conditioning step. The pH modification process, which is an integral part of alkyl-silica sorbent based SPE methods, can also affect method efficiency since analyte retention will be weak if the pH is not optimized. Alteration of the pH of the condition buffer solution was carried out by monitoring the extraction efficiency of rosiglitazone at pH 1–12. Triplicate analyses were performed for different pH conditions. Acidification of condition solution is likely to increase the dissociation of the analyte (Fig. 3); this may lead to decreasing extraction efficiency of rosiglitazone. The extraction efficiency of C8 sorbent increased significantly when the pH increased from 1 to 8 and to reach maximum at pH 8 and, then, decreased by increasing the basicity. Since pK_a value of rosiglitazone is between 6 and 7, at the working pH of 8, rosiglitazone shift significantly to the neutral form, which has a higher affinity for the C8 sorbent, thereby increasing the amount extracted. The amounts of rosiglitazone detected dropped about 30% upon increased basicity from pH 8 to pH 12. Consequently, a 10 mM ammonium acetate buffer with pH 8 was selected in this work. This nearly neutral pH is expected to prevent the dissociation of rosiglitazone, providing the best analytical performance in SPE.

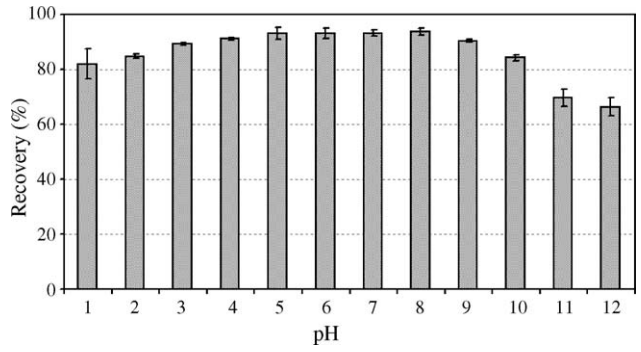

Fig. 3. The effect of pH on the recovery of 10 ng/mL rosiglitazone in urine.

It is well known that most compounds present in the sample are retained on the sorbent because non-specific hydrophobic interactions dominate under aqueous loading conditions. Different proportions of methanol–water were investigated to wash the cartridge and discard the endogenous components retained in the sorbent. A washing (clean-up) step was done with either water or 1–10% methanol–water. Higher recovery was obtained when the sorbent was washed with 1 mL 100% water, whereas increased proportion of methanol–water was unsuitable for washing, because analyte breakthrough was observed with the ratios from 1 to 10% of methanol. Furthermore, the absence of a washing step was disadvantageous for the LC column behavior and life-time by yielding unclean extracts. Thus, 1 mL of water for washing was chosen to gain cleaner extracts without losing the recovery.

As a general rule, the recovery of organic compounds by SPE is highly dependent on the polarity of the eluent. Since the elution solvent used in the current method must be compatible with LC–MS, the present method avoids the use of solvent such as hexane and dichloromethane, which are water immiscible and present problems with aqueous based SPE protocols. In this assay, different organic solvents and mixed solvents including, acetonitrile, methanol, ethanol, acetonitrile/methanol (1:1), acetonitrile/ethanol (1:1) or methanol/ethanol (1:1), were used for studying elution efficiencies of rosiglitazone from urine. Results showed that a simple one solvent elution system using acetonitrile was significantly better than that obtained for others. Consequently, after the comparison based on elution recovery and sensitivity, acetonitrile was selected. In addition, the eluotropic strengths of the elution solvents were adjusted with variation of the hydrochloric acid and acetic acid portion in the organic phase. We speculate that the addition of acid as a polar modifier to acetonitrile enabled the efficient elution of rosiglitazone from the sorbent. Nevertheless, our experiments indicated that the dissociated form of rosiglitazone was combined with both acids and produced salts. Even the residues were reconstituted with water, the compounds might be salting out in the LC eluent; hence, the acid eluting was not considered. The basicity of eluting was also determined by testing different concentrations of ammonium hydroxide at the elution step. The recovery of rosiglitazone was decreased by increasing the basicity of eluting, probably due to the affecting of analyte dissociation and its solvability in elution solvent; therefore, the adjustment of the elution solvent was not considered further. An elution profile of the content (%) of rosiglitazone in successive 1 mL aliquots of elution solvent was developed to determine the effect of acetonitrile volume on the elution efficiency of analyte from the cartridge. Rosiglitazone in each 1 mL fractions (eluate) 1–5 was analyzed (Table 3). Recovery (%) of rosiglitazone in the fractions 1–2 was over 99. It was found that complete elution of rosiglitazone from the cartridge was obtained within 3 mL of

Table 3
Effect of acetonitrile volume on the elution of 10 ng/mL rosiglitazone from the C8 cartridges

Fraction (eluate volume: 1 mL)	Recovery (%)	
	Mean	SD ($n = 3$)
1	97.0	0.05
2	2.5	0.06
3	0.5	0.13
4	0	–
5	0	–

elution solvent (fractions 1–3). To ensure a quantitative elution, 3 mL was used in final eluting.

To investigate the effect of drying step before elution, drying times of 0–5 min were tested. Results showed that the efficiency of extraction did not vary when the drying period was varied from 0 to 5 min for rosiglitazone. Since the acetonitrile is used for elution, the solvent will be miscible with water in the silica matrix, and then interact with all areas of the sorbent and not be stopped by residual water trapped in the pores. Hence, the drying step was not necessary. This extraction procedure was entire optimized to reduce the labor-intensiveness and analyst time required. As a consequence, the robustness of the method makes it easy for an operator to learn the technique quickly and to generate reproducible results.

3.6. Assay validation

The much higher selectivity of APCI-MS/MS detection was allowed the development of a very specific and rapid method for the determination of rosiglitazone in urine. Representative chromatograms of blank urine and urine spiked with rosiglitazone at the concentration of 0.1 ng/mL are shown in Fig. 4. The mass chromatogram of blank urine sample is clean and free from endogenous interferences at the retention time of rosiglitazone. Consequently, the proposed method was demonstrated to have the high specificity. The peak areas of the ion m/z 135 were extracted for quantification through the calibration curve of rosiglitazone. Calibration curve was established at seven different concentrations by LC–APCI-MS/MS in SRM mode. The assay was found to be linear in the range 0.05–500 ng/mL with a squared correlation coefficient (r^2 value) of 0.9993. Under the experimental conditions, the LOQ for rosiglitazone was 0.1 ng/mL. Based on the lowest detectable peak with a signal-to-noise ratio of 3, the LOD was found to be 0.03 ng/mL which is lower than that for the previously method reported.

Intra-day precision and accuracy were evaluated by analyzing quintuplicate samples of QC urine at five concentration levels (0.1, 1, 10, 50 and 100 ng/mL) in the same day. The precision of the method was calculated as the relative standard deviation (RSD). The RSDs for the intra-day precision ranged from 1.7 to 9.8% as shown in Table 4. Inter-day precision and accuracy were determined at the same five concentrations analyzed over a period of three days by preparing the fresh calibration curves in triplicate analyses each day. The RSDs for the inter-day precision ranged from 2.6 to 7.9% (Table 4). The accuracies were determined by comparing the mean calculated concentration of the spiked urine samples with the target concentration. The intra- and inter-day accuracies for all the samples were found to be within 91.0–103.6%. These results indicated that even without internal standard, the repeatability of the accurate assay was acceptable and comparable with other techniques using internal standard for improving precision [18,22,23].

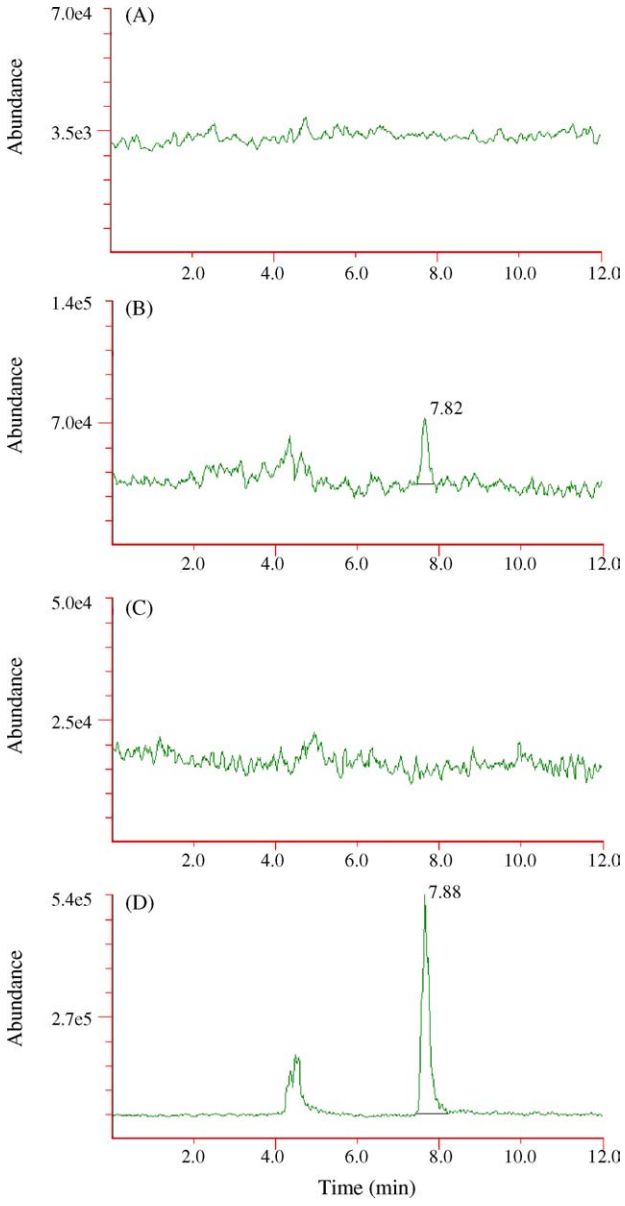

Fig. 4. Mass ion chromatograms for (A) blank urine sample; (B) LOQ, 0.1 ng/mL of rosiglitazone in human urine; (C) predose urine sample from the volunteer; (D) 1 h post-dose urine sample from the volunteer following a 4 mg single oral dose of Avandia®, produced by using SRM.

Ion suppression of ionization was evaluated by comparing the absolute peak areas of control urine (drug-free) extracted and then spiked with a known amount of drug, to neat standard injected directly in the same reconstitution solvent. It was shown that SPE improving the sample clean-up to remove urine components and thereby decreasing the amount of matrix injected onto the column, thus the ion suppression effect was minimized.

Table 4
Intra- and inter-day precision and accuracy of the QC samples of rosiglitazone in urine

Spiked concentration (ng/mL)	Intra-assay ($n = 5$)			Inter-assay ($n = 9$)		
	Mean ± SD	RSD (%)	Accuracy (%)	Mean ± SD	RSD (%)	Accuracy (%)
0.1	0.104 ± 0.010	9.8	103.6	0.091 ± 0.004	4.9	91.0
1.0	0.99 ± 0.02	2.5	99.2	1.00 ± 0.08	7.9	100.3
10.0	9.96 ± 0.28	2.8	99.6	10.0 ± 0.58	5.8	100.0
50.0	50.5 ± 1.0	2.0	101.0	50.6 ± 1.5	3.0	101.2
100	99 ± 2	1.7	99.1	100 ± 3	2.6	99.7

Table 5
The recovery data for assays of rosiglitazone in urine

Spiked concentration (ng/mL)	Measured concentration (ng/mL)	RSD (%)	Recovery (%)
0	0.092 ± 0.005	5.4	92.0
1.00	0.91 ± 0.05	5.5	91.2
10.0	9.9 ± 0.6	6.0	99.3
50.0	49.2 ± 0.7	1.4	98.5
100	98.6 ± 4.6	4.7	98.6

Data are mean ± SD of triplicate determinations.

Table 5 shows the absolute recoveries for the method developed, using laboratory-packed cartridges for QC urine spiked at five different concentration levels. The recoveries of extraction were calculated by comparing the peak areas obtained from the extract of the spiked urine sample with those obtained by direct injection of standard solutions. The average procedural recoveries for rosiglitazone were greater than 91.2% at all five concentrations tested, indicating no significant analytes loss derived from matrix effects in urine samples.

4. Application

After a single oral dose of 4 mg of Avandia® to one healthy female volunteer, the urine concentration of rosiglitazone was determined by the LC–APCI-MS/MS method described. The SPE was operated at the optimum conditions studied. Three replicate analyses were performed for each time point. Representative SRM chromatograms of urine sample collected from the volunteer, at 1 h, the time of C_{max} (2.78 ng/mL), after a single oral dose of 4 mg Avandia® and before administration are shown in Fig. 4. Fig. 5 shows the urinary elimination profile of rosiglitazone in the healthy volunteer. The results indicated that only a small amount of the administered dose was recovered as parent drug with peak urinary concentration at 2.78 ± 0.08 ng/mL (mean ± SD) after 1 h post-administration. This observation is showing below 0.1% excretion of parent rosiglitazone in urine consistent with reported of human studies on rosiglitazone [12]. After 6 h post-administration, the urine concentration of rosiglitazone was below ng/mL, and therefore, not possible to determine by the previous assay methods. Rosiglitazone had peak concentration (2.78 ng/mL) at 1 h post-administration and could be detected for up to 10 h (0.087 ng/mL), suggesting that urine may provide longer detection duration than plasma [10,11]. This study also examined the effectiveness of the proposed method for practical application by analyzing the actual specimens obtained from diabetic patients who were underwent therapeutic doses of rosiglitazone in a hospital. Representative chromatograms of the

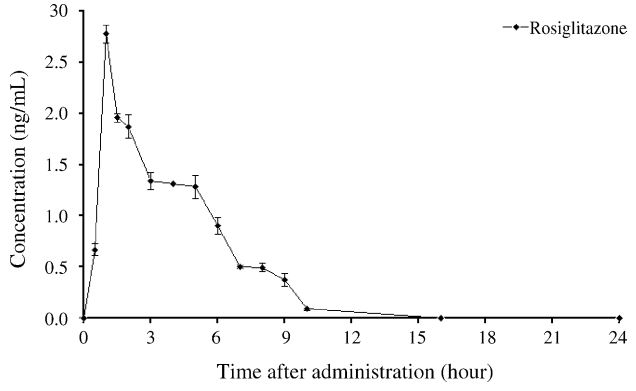

Fig. 5. Urinary elimination of rosiglitazone from the volunteer after an oral administration of 4 mg of Avandia®. Data points represent mean ± standard deviation, $n = 3$ analyses per time point.

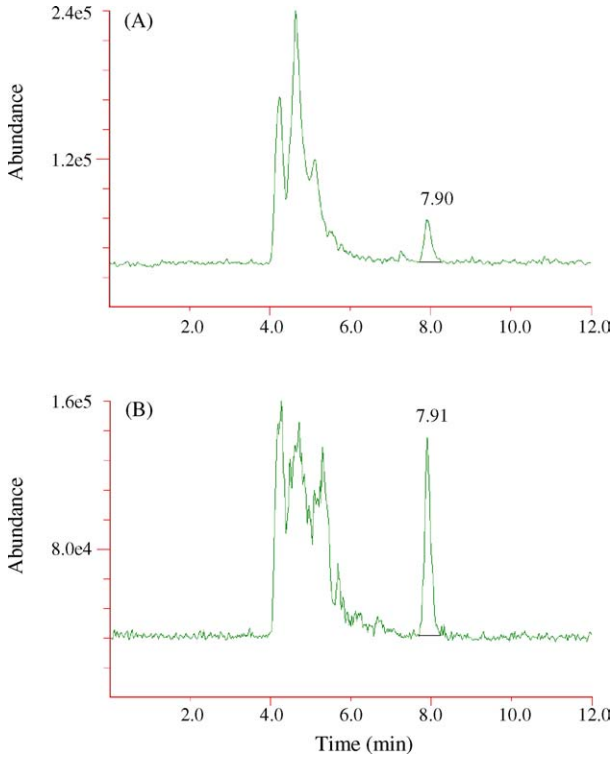

Fig. 6. Mass ion chromatograms of the urine samples taken from the rosiglitazone dosed patient S_1 (A) and patient S_2 (B), produced by using SRM.

Table 6
Rosiglitazone concentrations of urine samples from rosiglitazone dosed diabetic patients

Subject	Rosiglitazone (pg/mL)[a]
S_1	164 ± 11
S_2	760 ± 26
S_3	254 ± 9
S_4	280 ± 10

[a] Triplicate measurements.

urine samples taken from two rosiglitazone dosed patients are shown in Fig. 6. Table 6 indicates rosiglitazone was determined in real urine samples by using SPE and LC–MS/MS. According to the results, rosiglitazone was found in all urine samples. Rosiglitazone was detected ranging from 760 to 164 pg/mL. The results obtained from actual case studies clearly indicate that this method is sensitive enough to be used for practical trace drugs investigations. Additionally, the small volume of sample used for this method can be very useful when biological samples are limited.

5. Conclusion

This study evaluated the coupling of SPE with LC–APCI-MS/MS to determine the trace amount of rosiglitazone in urine samples. The total analytical time was less than 18 min including sample preparation. The procedure is based on isolation of rosiglitazone from urine using a SPE cartridge of 50 mg C8 sorbent, with satisfactory recovery and specificity. Quantification limit of 0.1 ng/mL of rosiglitazone in urine was achieved and better than those by the previous methods reported. This method was validated through its application to the analysis of urine samples obtained from drug administration studies. After an oral dosing with 4 mg of rosiglitazone, the peak urine concentration of rosiglitazone was rapidly attained at 1 h, and detected for up to 10 h. In addition, the feasibility of applying the methods to determine rosiglitazone in real samples was also examined by analyzing urine samples from rosiglitazone dosed patients. Rosiglitazone was detected ranging from 760 to 164 pg/mL. Due to the achieved results, this method can be easily modified, and then quantified other glitazone drugs. In view of the simplicity, sensitivity and selectivity, the present method is recommendable for the analysis of rosiglitazone in biological and pharmacological research, rendering it valuable for diagnostic purposes.

Acknowledgments

Financial support from National Science Council of Taiwan under contract no. NSC 91-2113-M-005-025 and National Chung-Hsing University under contract no. TCVGH-NCHU927610 is acknowledged.

References

[1] P.W. Young, D.R. Buckle, B.C. Cantello, H. Chapman, J.C. Clapham, P.J. Coyle, D. Haigh, R.M. Hindley, J.C. Holder, H. Kallender, A.J. Latter, K.W.M. Lawrie, D. Mossakowska, G.J. Murphy, L. Roxbee Cox, S.A. Smith, J. Pharmacol. Exp. Ther. 284 (1998) 751.
[2] J. Patel, E. Miller, R. Patwardhan, Diabetes 47 (Suppl. 1) (1998) 17.
[3] J.M. Lehmann, L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson, S.A. Kliewer, J. Biol. Chem. 270 (1995) 12953.
[4] T.M. Willson, J.E. Cobb, D.J. Cowan, R.W. Wiethe, I.D. Correa, S.R. Prakash, K.D. Beck, L.B. Moore, S.A. Kliewer, J.M. Lehmann, J. Med. Chem. 39 (1996) 665.
[5] F. Ovalle, J.F. Ovalle-Berúmen, South Med. J. 95 (2002) 1188.
[6] C. Ju, J.P. Uetrecht, Curr. Drug Metab. 3 (2002) 367.
[7] L.V. Gaal, A.J. Scheen, Diabetes Metab. Res. Rev. 18 (2002) S1.
[8] R.R. Henry, Endocrinol. Metab. Clin. North Am. 26 (1997) 553.
[9] J.M. Lehmann, L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson, S.A. Kliewer, J. Biol. Chem. 270 (1995) 12953.
[10] A.K. Miller, A.L. Inglis, K. Thompson, C.C. Davie, S. Schenker, R. Blum, D. Jorkasky, M.I. Freed, Clin. Pharmacol. Ther. 65 (2) (1999) 186.
[11] M.I. Freed, A. Allen, D.K. Jorkasky, R.A. DiCicco, Eur. J. Clin. Pharmacol. 55 (1999) 53.
[12] P.J. Cox, D.A. Ryan, F.J. Hollis, A.M. Harris, A.K. Miller, M. Vousden, H. Cowley, Drug Metab. Dispos. 28 (7) (2000) 772.
[13] T. Radhakrishna, J. Satyanarayana, A. Satyanarayana, J. Pharm. Biomed. Anal. 29 (2002) 873.
[14] P. Gomes, J. Sippel, A. Jablonski, M. Steppe, J. Pharm. Biomed. Anal. 36 (2004) 909.
[15] B.L. Kolte, B.B. Raut, A.A. Deo, M.A. Bagool, D.B. Shinde, J. Chromatogr. Sci. 42 (2004) 70.
[16] B.L. Kolte, B.B. Raut, A.A. Deo, M.A. Bagool, D.B. Shinde, J. Chromatogr. B 788 (2003) 37.
[17] M.H. Gaffney, G.D. Allen, R.W. Abbott, N.J. Deeks, F.J. Bollis, G. Rhodes, Methodol. Surv. Bioanal. Drugs 23 (1994) 251.
[18] A.M. Muxlow, S. Fowles, P. Russell, J. Chromatogr. B 752 (2001) 77.
[19] R.N.V.S. Mamidi, B. Benjamin, M. Ramesh, N.R. Srinivas, Biomed. Chromatogr. 17 (2003) 417.
[20] K.A. Kim, J.Y. Park, Biomed. Chromatogr. 18 (2004) 613.
[21] M.W. Hruska, R.F. Frye, J. Chromatogr. B 803 (2004) 317.
[22] Z.J. Lin, D. Desai-Krieger, L. Shum, J. Chromatogr. B 801 (2004) 265.
[23] N.M. Ho, C.H. Yiu, S.M. Wan, B.D. Stewart, K.L. Watkins, J. Chromatogr. B 811 (2004) 65.
[24] J.R. Soglia, S.P. Harriman, S. Zhao, J. Barberia, M.J. Cole, J.G. Boyd, L.G. Contillo, J. Pharm. Biomed. Anal. 36 (2004) 105.
[25] R. Majors, D. Raynie, LC–GC 15 (12) (1997) 1106.
[26] C. Müeller, P. Schäfer, M. Störtzel, S. Vogt, W. Weinmann, J. Chromatogr. B 773 (2002) 47.
[27] S. Briem, E. Eklund, G. Williams, J. Brunvall, Chromatographia 55 (2002) 35.
[28] E.P. Hunter, S.G. Lias, J. Phys. Chem. Ref. Data 27 (1998) 413.
[29] T.L. Constantopoulos, G.S. Jackson, C.G. Enke, J. Am. Soc. Mass Spectrom. 10 (1999) 625.
[30] L. Tang, P. Kebarle, Anal. Chem. 63 (1991) 2709.
[31] Y. Hsieh, G. Wang, Y. Wang, S. Chackalamannil, J.M. Brisson, K. Ng, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 16 (2002) 944.
[32] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.