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Development of a rapid UPLC-MS/MS method for quantification of saxagliptin in rat plasma and application to pharmacokinetic study

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ABSTRACT: A novel, simple and rapid ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) assay was established for quantification of saxagliptin in rat plasma. Plasma samples were processed by liquid–liquid extraction with ethyl acetate and chromatographed on a C_{18} column (2.1 × 50 mm i.d., 1.7 μm). The mobile phase consisted of methanol and 0.1% formic acid (40:60, v/v). Multiple reaction monitoring transitions were performed for detection in positive-ion mode with an electrospray ionization source. The calibration curve was linear over the concentration range of 0.5–100 ng/mL ($R^2 > 0.99$). All accuracy values were between 90.62 and 105.60% relative error and the intra- and inter-day precisions were less than 9.66% relative standard deviation. Extraction recovery was more than 81.01% and the matrix effect ranged from 90.27 to 109.15%. After validation, the method was applied to a pharmacokinetic study where healthy rats were orally given 0.5 mg/kg saxagliptin.

Keywords: UPLC-MS/MS; saxagliptin; method validation; pharmacokinetic application

Introduction

Saxagliptin (also known as BMS-477118 earlier, structure in Fig. 1) has been designed as a highly potent and selective inhibitor against dipeptidyl peptidase-4 (DPP-4) for treatment of type 2 diabetes mellitus (T2DM; Gallwitz, 2008). Inhibition of DPP-4 is a new and promising mechanism for T2DM treatment. It mitigates DPP-4-mediated inactivation of glucagon-like peptide-1 (GLP-1), an insulinotropic hormone that stimulates insulin secretion in a glucose-dependent manner (Holst and Deacon, 1998). As such, DPP-4 inhibitors, including saxagliptin, can augment postprandial insulin secretion and improve the overall glycemic profile in T2DM patients (Deacon, 2007). Compared to other existing antidiabetic agents, DPP-4 inhibitors pose lower risks of hypoglycemia and weight gain owing to the mechanism that GLP-1 stimulates insulin secretion in a glucose-dependent manner (Gallwitz, 2008).

Saxagliptin was approved in 2009 by the US Food and Drug Administration, and has been marketed in China since 2011. In the Chinese T2DM patient population, concomitant uses of chemical drugs and herbal medicines are not uncommon. Given that saxagliptin is mainly metabolized by CYP3A4 (Bristol-Myers Squibb, 2003; Scheen, 2010) and that Chinese herbal medicines may modulate CYP3A4 activities (Hellum *et al*., 2007; Izzo, 2004; Modarai *et al*., 2010; Taesotikul *et al*., 2011; Wang *et al*., 2010), herb–drug interaction is reasonably expected. Therefore, laboratory and clinical research in this regard is imperative. With the long-term goal of establishing an approach for predicting and identifying herb–drug interaction, our group has launched an investigation into the potential interaction between saxagliptin and common antidiebetic herbal medicines. While analytical methods for quantification of saxagliptin in biological matrices have been reported, detailed information on sample preparation and method validation, to our knowledge, has not been fully disclosed (Fura *et al*., 2009; Patel *et al*., 2010; Upreti *et al*., 2011). Here, we present our development and validation of an UPLC-MS/MS method for plasma saxagliptin quantification. This method requires only a small sample volume (50 μL) and a short run time (1.2 min). The method was employed in a pharmacokinetic study where a dose of 0.5 mg/kg saxagliptin was administered to healthy rats. Our results suggest that this rapid, simple and sensitive method will enable our further study with the desired cost-effectiveness.

Experimental

Chemicals and reagents

Saxagliptin and sitagliptin (internal standard, IS) with purity >98% were obtained from Zhengzhou Huawen Chemical Co. Ltd (Hebei, China). HPLC-grade methanol and ethyl acetate were purchased from Tedia Company Inc. (Fairfield, OH, USA) and Honeywell Burdick & Jackson (Muskegon, MI, USA), respectively. Formic acid was from Chengdu Kelong Chemical Reagent Factory (Sichuan, China). Deionized water up

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Abbreviation used: ESI, electrospray ionization; MRM, multiple reaction monitoring; UPLC-MS/MS, ultraperformance liquid chromatography/tandem mass spectrometry; $T_{1/2}$, half-life; T_{max}, peak time; C_{max}, maximum concentration; AUC_{0-t}, area under the curve from time 0 the last time detected; Vz/F, apparent volume of distribution; Cl/F, clearance.

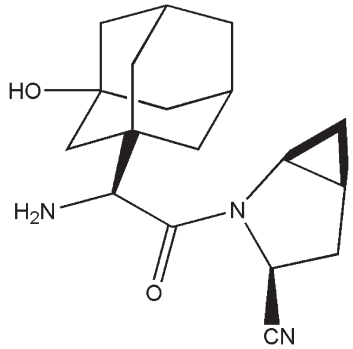

Figure 1. Chemical structure of saxagliptin.

to a resistivity of 18.2 MΩ was purified with an Elga water purification system (London, UK).

Instrumentation and analytical conditions

Sample analysis was performed on an UPLC-MS/MS system. An Acquity™ UPLC™ system (Waters Corporation, Milford, MA, USA) was composed of an autosampler (set at 4 °C), a column oven (set at 45 °C) and a binary solvent delivery manager. A tandem mass spectrometry was used for detection.

Chromatographic separation was achieved on a Waters Acquity BEH C_{18} column (2.1 × 50 mm i.d., 1.7 μm, Waters, Wexford, Ireland) preceded by a BEH C_{18} VanGuard™ pre-column (2.1 × 5 mm i.d., 1.7 μm, Waters, Wexford, Ireland). An isocratic elution lasting 1.2 min was obtained; the mobile phase contained 40% methanol and 60% 0.1% formic acid in water and was pumped at 0.35 mL/min. A typical injection volume was 10 μL.

The detection system, a tandem quadrupole mass spectrometer (Waters Quattro Premier XE, Micromass MS Technologies, Manchester, UK), was operated in positive electrospray ionization (ESI^+) mode. Multiple-reaction monitoring (MRM) mode was selected for quantification of saxagliptin and IS, for which the precursors to production ion transitions were as follows: saxagliptin, 316.1 → 179.9; IS, 408 → 234.9 (detection span was set at 0.5 *m/z*). Cone voltage and collision energy were respectively 30 and 18 V for both compounds. Ultra-high pure nitrogen and argon were used as desolvation gas (700 L/h) and collision gas (0.21 mL/min), respectively. Masslynx™ 4.1 software was used to collect and process data.

Preparation of calibration standards and quality control samples

Standard master stock solutions of saxagliptin (159.6 μg/mL) and IS (181.2 μg/mL) were obtained by dissolving an appropriate amount of saxagliptin and IS in methanol. A series of working standard solutions for saxagliptin and quality control (QC) spiking solutions were prepared by serial dilution of the respective standard master stock solutions in methanol.

Aliquots of the working standard solutions for saxagliptin were spiked with blank rat plasma to yield seven calibration standards ranging from 0.5 to 100 ng/mL. The quality control standards at 1 ng/mL (low), 10 ng/mL (medium) and 100 ng/mL (high) were prepared in the same manner. A working IS solution of 70 ng/mL was prepared by appropriate dilution of the IS stock solution in water.

Sample extraction

A simple liquid–liquid extraction was applied for saxagliptin extraction from plasma samples. An aliquot of the rat plasma sample (50 μL) was added with 20 μL IS solution and then extracted with 1 mL ethyl acetate via vortex-mixing for 2 min. After centrifugation, 800 μL of the organic supernatant was evaporated to dryness at room temperature under a gentle stream of air followed by reconstitution with 200 μL mobile phase. A 10 μL processed sample was injected for analysis.

Method validation

The method was validated in terms of selectivity, linearity, accuracy, precision, extraction recovery, matrix effect and stability according to US Food and Drug Administration guidelines for bioanalytical method validation (US Food and Drug Administration, 2001).

Selectivity and specificity

This step was carried out to identify potential chromatographic interference from endogenous entities at the peak regions of the analyte and IS. Chromatographic peaks from plasma samples were compared with the authentic standards by the retention times and MRM responses.

Linearity and LLOQ

The seven-point linear calibration curve was constructed using weighted ($1/x^2$) quadratic regression by plotting the peak area ratio of saxagliptin to IS vs the theoretical plasma concentrations over the range of 0.5–100 ng/mL. The lower limit of quantitation (LLOQ) was defined as the concentration where the accuracy and precision were up to ±20% relative error (RE, %) and relative standard deviation (RSD, %), respectively.

Accuracy and precision

Accuracy and intra- and inter-day precision were assessed by analyzing three consecutive batches containing calibration curve standards and five replicates of each QC level (low, medium and high). The accuracy was expressed as RE (%), and the precision as RSD (%). Both RE and RSD were expected to be within ±15% to be acceptable.

Extraction recovery

Extraction recovery was assessed by comparing the peak responses from QC samples with those from unextracted samples. Unextracted samples were prepared as follows: adding 200 μL standard solution into extracted blank plasma to yield equivalent concentrations to corresponding QC samples. Recovery of saxagliptin was tested at low, medium and high QC concentrations whereas that of IS was tested only at the concentration added. Samples in each concentration were in triplicate.

Matrix effect

Whether or not plasma endogenous constituents influence the ionization of saxagliptin and IS was determined by means of matrix effect, which was measured via comparison of peak response from unextracted samples with that from neat samples at equivalent concentrations. Concentration levels and amount of samples were identical to those in the recovery test.

Stability

Three aliquots of low and high QC samples were tested for pre-treatment, post-treatment, three cycles of freeze–thaws and long-term stabilities of the analyte. Pre-treatment stability was assessed by leaving QC samples at room temperature for 4 h before extraction. For post-treatment assessment, all QC samples were placed in 4 °C for 24 h before analysis. A freeze–thaw cycle plasma stability test was carried out via repeatedly freezing and thawing QC samples for three cycles before treatment. Long-term stability was evaluated by storing QC samples at −20 °C for 7 days before thawing and extraction.

Pharmacokinetic application

The study was approved by the Animal Ethics Committee of Sun Yat-Sen University. Six SPF-grade Wistar male rats weighing 220–230 g (Certificate No. SCXK2011-0029) were provided by Sun Yat-Sen University Laboratory Animal Center.

All rats were fasted but with free access to water for 12 h prior to the pharmacokinetic study. Each rat was given a single oral dose of 0.5 mg/kg saxagliptin (corresponding to the highest recommended dose in clinic, 5 mg once a day) suspended in 5 g/L CMC-Na. Blood samples were drawn into heparinized tubes at 10, 20, 30, 50, 70 min and 1.5, 2, 3, 5, 8 h post-dose. A 50 μL aliquot of plasma was separated from each collection and stored at −20 °C until analysis. Winnonlin 5.0.1 was used to estimate pharmacokinetic parameters.

Results and discussion

Liquid chromatography and extraction

Methanol elution improved peak shapes and the proposed percentage (40%) in the mobile phase gave consideration to both retention times and peak widths. Acetonitrile was not chosen for the mobile phase for several reasons. First, at ≥30% acetonitrile in the mobile phase (isocratic elution), the compounds, especially saxagliptin and vildagliptin (taken as IS initially, but abandoned owing to instability after reconstitution), formed peaks at two retention times, one at a dead time and the other at a real retention time (Fig. 2. A); if we tried to lower the proportion of acetonitrile, saxagliptin formed one normal peak but vildagliptin still had two peaks, and sitagliptin also began to show a tailing tendency (Fig. 2B). Therefore, acetonitrile was not selected.

Saxagliptin has a very high pK_a (7.3; Hess *et al*., 2011) and is polar owing to the existence of amino and especially cyano groups. Therefore ethyl acetate and another relative polar

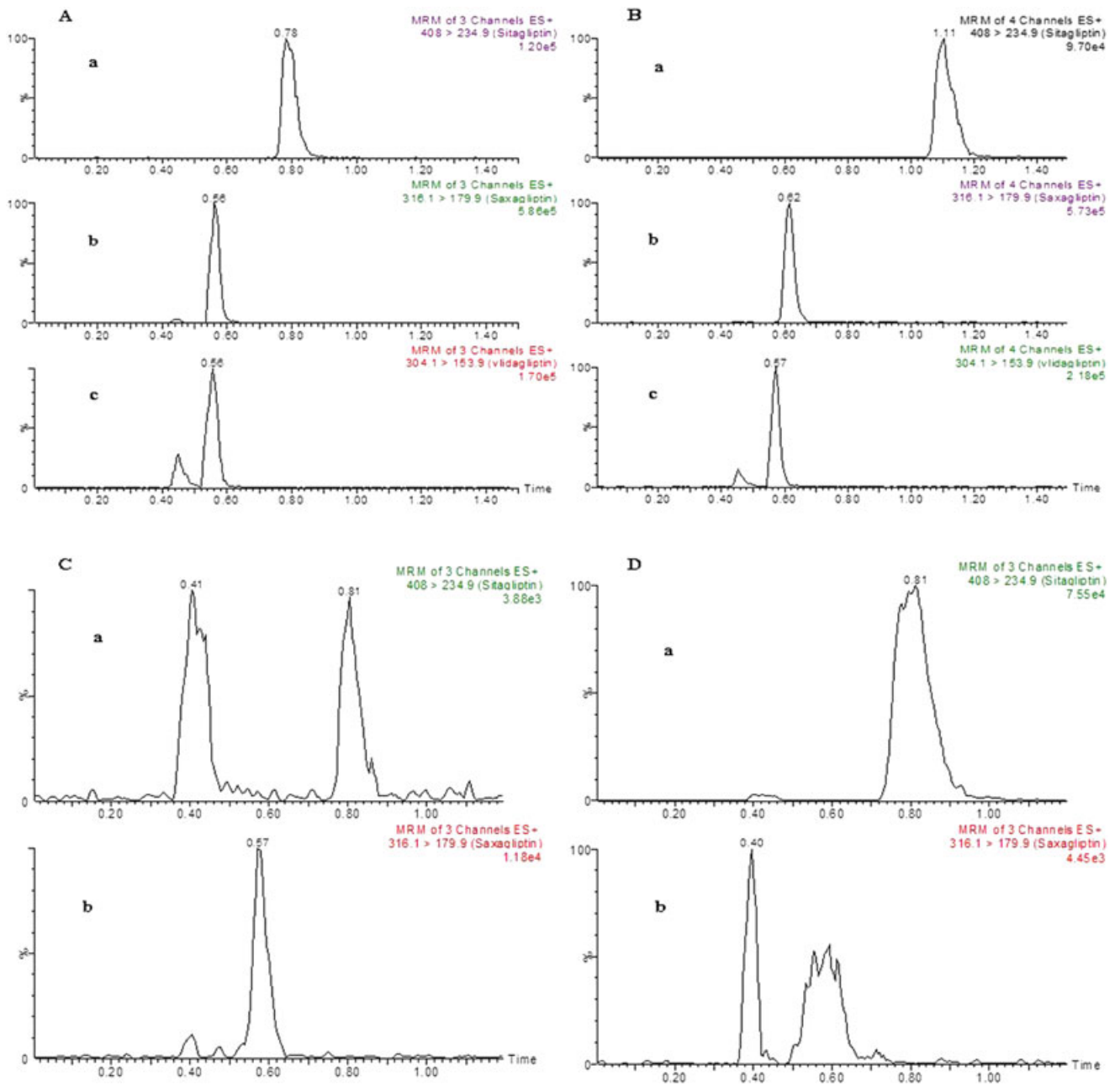

Figure 2. Chromatograms of isocratic elution with 30% acetonitrile in mobile (A), 25% acetonitrile in mobile (B), sample extracted with acetone (C) and IS diluted in methanol (D). Channel a, sitagliptin; b, saxagliptin; c, vildagliptin.

medium, acetone, were tested for extraction. Acetone extraction resulted in two peaks in the IS channel (Fig. 2C) while ethyl acetate did not. Ethyl acetate extracted both analytes satisfactorily and yielded specific and typical symmetrical peaks.

IS stock solution was diluted with water rather than methanol before addition to samples. The dilution in methanol caused both compounds to peak at two retention times as mentioned above (Fig. 2D), which was prevented by dilution in water.

Selectivity and specificity

Retention times of saxagliptin and IS were 0.55 and 0.76 min, respectively. No significant interferencing peaks were observed in the retention regions of both compounds. Figure 3 shows typical chromatograms from blank plasma, plasma spiked with saxagliptin at the LLOQ, and plasma obtained 1.5 h after administration.

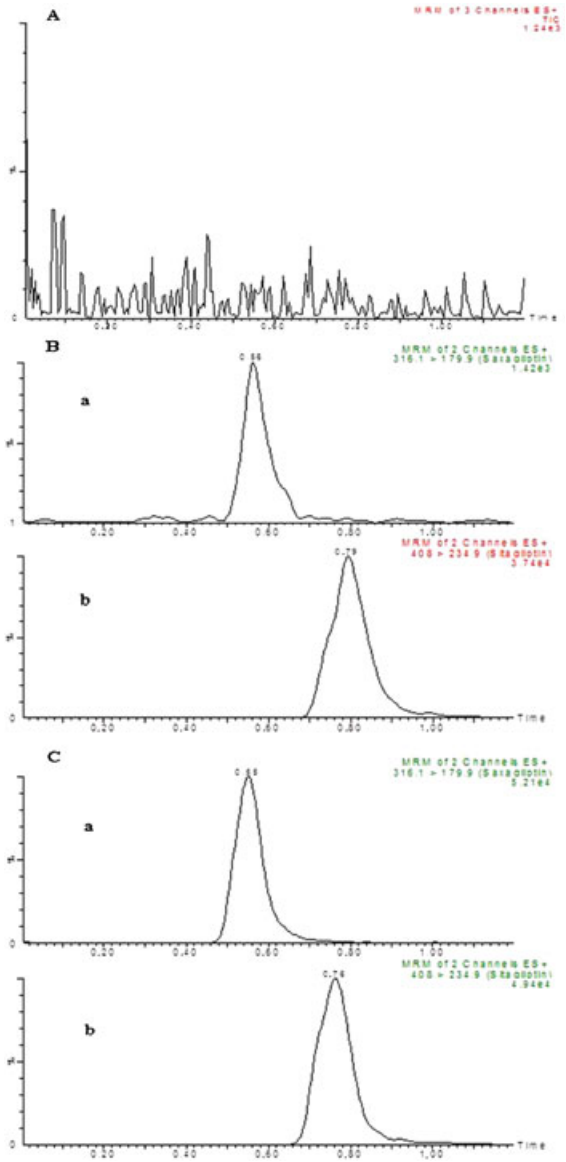

Figure 3. Chromatograms of blank rat plasma (A), blank rat plasma spiked with saxagliptin at LLOQ (0.5 ng/mL) (B), and plasma sample from a rat at 1.5 h after oral administration of 0.5 mg/kg saxagliptin (C). Channel a, saxagliptin; b, IS.

Linearity, accuracy and precision

Correlation coefficients (R^2) of calibration curves in all inter-run cases were >0.99 over the concentration range from 0.5 to 100 ng/mL. A weight of $1/x^2$ was applied to minimize the relative error for the curve fitting. The LLOQ of saxagliptin was 0.5 ng/mL in this assay.

Tables 1 and 2 present the results for accuracy and precision evaluation. The results meet the pertinent guidelines. For saxagliptin, accuracy ranges from 90.62 to 105.60% (RE), and intra- and inter-day precisions were 3.06–8.51 and 4.89–9.66% (RSD), respectively. These results indicate that it is a precise and accurate method.

Extraction recovery

As detailed in Table 3, the overall recovery efficiency for saxagliptin was 96.43% at 1 ng/mL, 92.32% at 10 ng/mL and

Table 1. Intra-day precision data of saxagliptin

Quality control	Theoretical concentration (ng/mL) ($n = 5$)	Run	Measured concentration (ng/mL)			
			Mean	SD	RSD (%)	Accuracy (%)
LLOQ	0.5	1	0.50	0.04	8.01	100.81
		2	0.51	0.04	6.96	102.42
		3	0.53	0.03	5.90	105.60
Low QC	1	1	0.96	0.06	5.83	95.80
		2	0.96	0.06	6.49	95.82
		3	1.02	0.03	3.06	102.40
Mid QC	10	1	9.06	0.39	4.25	90.62
		2	9.32	0.58	6.25	93.23
		3	9.46	0.81	8.51	94.62
High QC	100	1	95.42	4.70	4.93	95.42
		2	101.97	5.44	5.34	101.97
		3	103.61	5.95	5.74	103.61

QC, Quality control; LLOQ, lower limit of quantification.

Table 2. Inter-day precision data of saxagliptin

Quality control	Theoretical concentration (ng/mL) ($n = 15$)	Measured concentration (ng/mL)			
		Mean	SD	RSD (%)	Accuracy (%)
Low QC	1	0.98	0.08	8.53	98.00
Mid QC	10	9.28	0.45	4.89	92.81
High QC	100	100.33	9.69	9.66	100.33

Table 3. Extraction recovery and Matrix effect data of saxagliptin and IS

	Saxagliptin			IS
	Low QC	Mid QC	High QC	
Extraction recovery ($n = 3$, mean ± SD, %)	96.43 ± 4.58	92.32 ± 3.11	81.01 ± 1.10	108.66 ± 1.23
RSD (%)	4.75	3.37	1.36	1.13
Matrix effect ($n = 3$, mean ± SD, %)	109.15 ± 3.42	95.08 ± 2.40	90.27 ± 0.99	99.67 ± 0.59
RSD (%)	3.13	2.52	1.09	0.59

81.01% at 100 ng/mL; the recovery for IS was 108.66%. The recovery result indicates that ethyl acetate is a feasible and appropriate medium for saxagliptin and sitagliptin extraction.

Table 4. Stability data of saxagliptin under four conditions

Quality control ($n = 3$)	Conditions	Measured concentration (ng/mL)			
		Mean	SD	RSD (%)	Accuracy (%)
Low QC	4 h bench-top	0.95	0.05	4.82	95.00
	24 h autosampler	1.05	0.05	1.93	105.37
	Three freeze–thaw cycles	0.98	0.03	2.77	97.90
	7 days at −20 °C	0.97	0.03	3.10	96.66
High QC	4 h bench-top	101.73	2.33	2.29	101.73
	24 h autosampler	100.11	4.77	4.77	100.11
	Three freeze–thaw cycles	100.14	2.95	2.95	100.14
	7 days at −20 °C	101.30	0.94	0.92	101.30

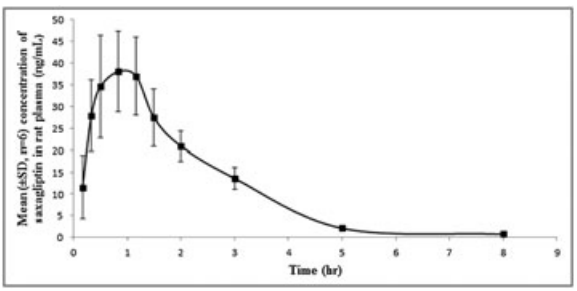

Figure 4. Mean (± SD, $n = 6$) concentration–time profile of saxagliptin in plasma after oral dosing of 0.5 mg/kg to rats.

Matrix effect

The matrix effect of QC samples at all three concentrations above was observed to be within the range 90.27–109.15% for saxagliptin and 99.67% for IS, indicating that matrix components did not significantly alter the performance of chromatography or the ionization of analytes; the matrix effect on the ionization of analytes was not serious under these experimental conditions and could be neglected.

Stability

As summarized in Table 4, the deviations of the measured concentrations from the standard ones in the stability tests were within the 15% assay variability limit. This result indicated that saxagliptin and IS were stable on the bench at room temperature for 4 h, during residence time in the auto-sampler, at the end of three consecutive freeze–thaw cycles and after long-term storage.

Pharmacokinetic application

Our UPLC-MS/MS method for saxagliptin quantification was employed in a pharmacokinetic study. Shown in Figure 4 is the concentration–time curve for plasma saxagliptin ($n = 6$) after a dose of 0.5 mg/kg in the rat. The mean concentration–time data were analyzed using noncompartmental analysis with the assistance of Winnonlin 5.0.1 software. The pharmacokinetic parameters are presented in Table 5. The half-life ($T_{1/2}$) is close to what Thareja *et al*. (2010) reported.

Conclusion

In this study, a novel, simple and rapid method for saxagliptin quantification was developed. The method possesses desired sensitivity, accuracy, precision and particularly a short analysis time. The applicability of this method was demonstrated in a pharmacokinetic study. This method will enable our further study on potential interactions between saxagliptin and common herbal medicines.

Table 5. Pharmacokinetic parameters in rats administered with 0.5 mg/kg saxagliptin

$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-t} (h ng/mL)	Vz/F (L)	Cl/F (L/h)
1.12 ± 0.03	0.90 ± 0.28	39.82 ± 7.72	84.78 ± 12.28	2.20 ± 0.37	1.42 ± 0.20

Data expressed as means ± standard deviation.

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