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Determination of sitagliptin in human urine and hemodialysate using turbulent flow online extraction and tandem mass spectrometry

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Abstract

High turbulence liquid chromatography (HTLC, or turbulent flow online extraction) and tandem mass spectrometry (MS/MS) methods for the determination of sitagliptin in human urine and hemodialysate were developed and validated to support clinical studies. A narrow bore large particle size reversed-phase column (Cyclone, 50 mm × 1.0 mm, 60 μm) and a BDS Hypersil C18 column (30 mm × 2.1 mm, 3 μm) were used as extraction and analytical columns, respectively. For the urine assay, the LLOQ was 0.1 μg/ml, the linear calibration range was 0.1 to 50 μg/ml, the interday precision (R.S.D.%, $n = 5$) was 2.3–6.5%, and the accuracy was 96.9–106% of the nominal value. For the urine quality control samples (QCs), the intraday precision (R.S.D.%, $n = 5$) and accuracy were 1.8–2.6% and 96.2–106% of the nominal value, respectively. The interday precision (R.S.D.%) for 56 sets of urine QCs over a 6-month period varied from 3.8% to 5.5% and the accuracy from 102% to 105% of the nominal value. For the hemodialysate assay, the LLOQ was 0.01 ng/ml, the linear dynamic range was 0.01–5.0 ng/ml, the interday precision was 1.6–4.1%, and the accuracy was 89.8–104% of the nominal value. For hemodialysate QCs, the intraday precision and accuracy varied from 2.3% to 8.9% and from 99.8% to 111% of the nominal value, respectively. These results demonstrated that both methods are selective, accurate, precise, reproducible, and suitable for quantifying sitagliptin in hemodialysate and human urine samples.

Keywords: Sitagliptin; Online extraction; LC–ESI-MS/MS; High turbulence liquid chromatography; Bioanalysis

1. Introduction

Sitagliptin [(2*R*)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6 dihydro [1,2,4]triazolo [4,3-*a*]pyrazin-7(8*H*)-yl] butan-2-amine] (Fig. 1) is an orally active, potent and selective inhibitor of dipeptidyl peptidase-IV (DPP-IV) for the treatment of Type 2 diabetes. DPP-IV inhibitors enhance levels of active GLP-1 and other incretins, and facilitate glucose-dependent insulin secretion [1]. To support clinical pharmacokinetic studies, a method for the determination of sitagliptin in human plasma has been developed and validated using HTLC/LC–MS/MS [2]. The pharmacokinetic and pharmacodynamic properties of single oral doses and multiple oral doses sitagliptin were investigated [3,4], and urinary sitagliptin concentrations were quantified. Sitagliptin was well absorbed with 75–80% of the oral dose being excreted unchanged in the urine of the subjects with normal renal function. In order to evaluate the pharmacokinetics of single doses of sitagliptin in patients with various degrees of renal insufficiency [5], assay was developed to quantify sitagliptin in hemodialysate samples.

High-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) is widely used for bioanalysis due to its selectivity and excellent sensitivity. An accurate and robust LC–MS/MS assay requires an effective sample cleanup procedure, such as solid-phase extraction (SPE) [6], liquid–liquid extraction (LLE) [7], or solid–liquid extraction (using diatomaceous earth) [8]. These optimized cleanup methods efficiently remove potentially interfering components (e.g. proteins, lipids, and salts), and provide a relatively clean sample for LC–MS/MS

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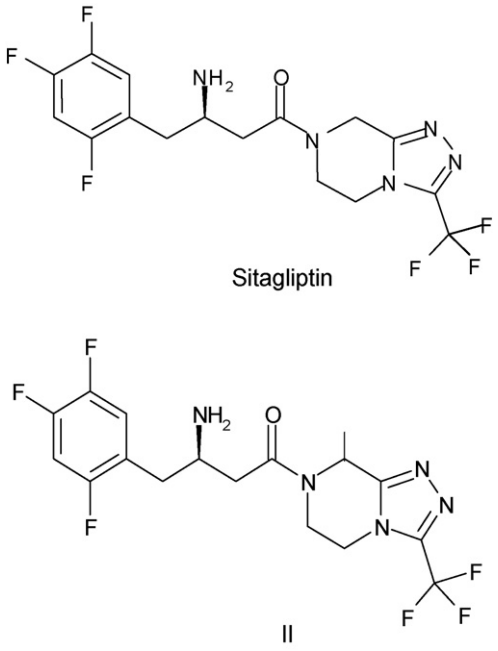

Fig. 1. Chemical structure of sitagliptin and internal standard (II).

analysis. However, these methods require multiple operation steps and long sample preparation time.

High turbulence liquid chromatography (HTLC) is a technique introduced by Cohesive Technologies (Franklin, MA, USA) in the late 1990s [9]. The major advantage of HTLC is that off-line time consuming sample cleanup is not necessary. The biological fluids can be injected directly onto an extraction column (50 mm × 1.0 mm) with a large particle size (50–60 μm). In this case, the sample matrix such as proteins and salts are rapidly eliminated to waste with a high flow rate (4–5 ml/min) aqueous mobile phase while small molecules are retained on the hydrophobic surface inside the porous particles [10–17]. With an organic mobile phase, the retained analytes are subsequently eluted from the extraction column onto an analytical column for chromatographic separation. HTLC/LC–MS/MS has been successfully employed for clinical sample analysis without off-line sample cleanup [2,18,19].

In this paper, we report assays for quantifying sitagliptin in human urine and hemodialysate samples. In addition, we present the validation, analyte stability, and matrix effect data for both assays.

2. Materials and methods

2.1. Materials

Sitagliptin and compound II (Fig. 1) were obtained from Merck Research Labs. (West Point, PA, USA). Compound II is the structure analogue of sitagliptin and was used as the internal standard (IS). The purity of sitagliptin and IS were 99.8% and 99.2%, respectively. HPLC grade acetonitrile (ACN), glacial acetic acid and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (98–100%) and

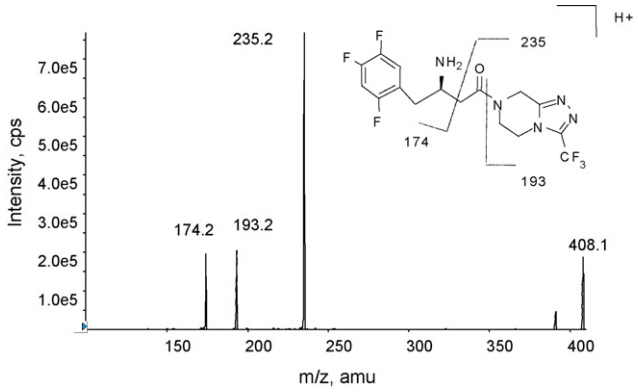

Fig. 2. Product scan spectra of sitagliptin.

ethylamine (70%) were purchased from Sigma (St Louis, MO, USA). Control urine was provided by our colleagues and control hemodialysate was purchased from Biological Specialty Corp. (Colmar, PA, USA).

2.2. Instruments

A Cohesive 2300 HTLC system which included a quaternary pump used as a loading pump, a binary pump used as an elution pump, and a valve module (Cohesive Technologies Inc., Franklin, MA, USA) was used for on-line extraction and separation. A LEAP HTS PAL autosampler (CTC Analytics, Zingen, Switzerland) was used for sample injection. A Packard MultiPROBE II HT EX liquid handling system (Meriden, CT, USA) was used in all pipetting steps during the sample preparation. Sciex API 4000 and API 365 mass spectrometers (Toronto, Canada) equipped with TurboIonSpray Interfaces were used as the detector for hemodialysate and urine assays, respectively. The data were collected and processed using Analyst software v. 1.4.1 (Sciex, Toronto, Canada).

2.3. Standard solutions and quality control (QC) samples

For the urine assay, the stock solutions of sitagliptin and IS were prepared at about 1.0 mg/ml in 50% ACN (ACN–water, 50:50, v/v). Working standard solutions from 0.1 to 50 μg/ml (0.1, 0.2, 0.5, 2, 5, 20, 40 and 50 μg/ml) in 10% ACN (ACN–water, 10:90, v/v) were prepared by serial dilution from the stock solution of sitagliptin. The working internal standard solution was prepared at 5.0 μg/ml in 10% ACN by dilution from the stock solution of IS.

For the hemodialysate assay, the primary stock solution of sitagliptin and IS were prepared at 100 μg/ml in 50% ACN. The secondary stock solutions of sitagliptin and IS were prepared at 1.0 and 0.5 μg/ml, respectively, by dilution from their primary stock solutions (100 μg/ml) with 10% ACN. Working standard solutions of sitagliptin from 0.1 to 50 ng/ml (0.1, 0.2, 0.5, 2.0, 5.0, 25, 40 and 50 ng/ml) in 10% ACN were prepared by serial dilution from the secondary stock solution (1.0 μg/ml) of sitagliptin. A working internal standard solution was prepared at 2.5 ng/ml by dilution from the secondary stock solution (0.5 μg/ml) of IS with 10% ACN.

Table 1
Online HTLC method for the urine assay

	Loading pump				Tee	Loop	Eluting pump		
	Time (s)	Flow (ml/min)	Grad	% B[a]			Flow (ml/min)	Grad	% B
Loading	30	5	Step	0		Out	0.4	Step	0
Transfer	72	0.2	Step	0	T	In	0.25	Step	0
Eluting	12	3	Ramp	30		In	0.4	Step	40
Eluting	36	3	Ramp	100		In	0.4	Ramp	70
Eluting	30	3	Ramp	70		In	0.4	Step	70
Eluting	18	3	Ramp	30		In	0.4	Ramp	100
Eluting	24	5	Step	30		In	0.4	Ramp	50
Equilibrate	6	2	Step	0		Out	0.4	Ramp	10

[a] Mobile phase A: 2.5 mM ethylamine, 0.1% formic acid (FA) aqueous solution; mobile phase B: 0.1% FA acetonitrile (ACN) solution.

Urine standards were prepared by mixing 100 μl of each working standard with 100 μl of control human urine, 50 μl of working IS solution, and 100 μl of 5% acetic acid in a 96-well plate. Hemodialysate standards were prepared by mixing 40 μl of each working standard with 400 μl of control hemodialysate, 40 μl of working IS solution, and 20 μl of 4% formic acid in a 96-well plate.

For the urine assay, a QC stock solution of sitagliptin, prepared from a separate weighing, was prepared at 1.0 mg/ml in 50% ACN. A secondary QC stock solution was prepared at 10 μg/ml in 10% ACN by dilution of the stock solution (1.0 mg/ml). QC samples were prepared by adding appropriate volumes of QC stock solutions (primary or secondary stock solutions) into volumetric flask and diluting to the mark with pooled control human urine to achieve the desired concentrations of 0.3, 5.0 and 40 μg/ml for low, medium and high QCs, respectively. 400 μl of each QC sample was transferred into conical polypropylene tubes which were capped and stored at −20 °C.

For the hemodialysate assay, a primary QC stock solution of sitagliptin, prepared from a separate weighing, was prepared at 100 μg/ml in 50% ACN. A secondary and a tertiary QC stock solution were prepared at 1.0 and 25 ng/ml by the serial dilution from the primary stock solution (100 μg/ml) with 10% ACN. QC samples were prepared by adding appropriate volumes of QC stock solutions (secondary and tertiary stock solutions) into volumetric flasks and diluting to the mark with pooled control hemodialysate to achieve the desired concentrations of 0.03, 0.5 and 4.0 ng/ml for low, medium and high QCs, respectively. 500 μl of each QC sample was transferred into polypropylene tubes which were capped and stored at −20 °C.

QC samples were processed along with the unknown clinical samples in each analytical batch. The number of QC samples in an analytical batch depended on the total number of samples analyzed in the batch. The minimum number of QC samples was at least 5% of the number of unknown samples analyzed in a given batch or six total QCs, whichever was greater [20].

2.4. Sample preparation

For the urine assay, a volume of 100 μl of QC or human clinical sample was transferred directly into a 96-well plate followed by 50 μl of working IS solution and 100 μl of 5% acetic acid. Then, 100 μl of 10% ACN was added to make up for the volume of the working standard. For the hemodialysate assay, a volume of 400 μl of QC or clinical hemodialysate sample was transferred directly into a 96-well plate followed by 40 μl of working IS solution and 20 μl of 4% formic acid. Then, 40 μl of 10% ACN was added to make up the volume.

2.5. Chromatographic conditions

For both urine and hemodialysate assays, a Cyclone HTLC column (50 mm × 1.0 mm, 60 μm particle size) from Cohesive Technologies Inc. (Franklin, MA, USA) and a BDS Hypersil C18 column (30 mm × 2.1 mm, 3 μm particle size) from ThermoHypersil-Keystone (Bellefonte, PA, USA) were used as

Table 2
Online HTLC method for the hemodialysate assay

	Loading pump				Tee	Loop	Eluting pump		
	Time (s)	Flow (ml/min)	Grad	% B[a]			Flow (ml/min)	Grad	% B
Loading	24	5	Step	0		Out	0.4	Step	0
Transfer	72	0.2	Step	0	T	In	0.25	Step	0
Eluting	12	5	Step	0		Out	0.4	Step	85
Eluting	30	2	Step	80		In	0.4	Step	85
Eluting	30	2	Ramp	10		Out	0.4	Step	85
Eluting	12	2	Ramp	80		In	0.4	Step	85
Eluting	12	2	Step	80		Out	0.4	Step	85
Eluting	24	5	Step	40		In	0.6	Step	85
Equilibrate	12	2	Ramp	5		Out	0.6	Ramp	5

[a] Mobile phases A and B: same as in Table 1.

extraction and analytical columns, respectively. Mobile phase A was 2.5 mM ethylamine, 0.1% formic acid (FA) aqueous solution; mobile phase B was 0.1% FA acetonitrile (ACN) solution. The injection volume was 5 and 40 μl for the urine and hemodialysate assay, respectively.

The focus mode online extraction method [2] was modified for sitagliptin urine and hemodialysate assays (Tables 1 and 2). The focus mode online extraction method consists of four general steps: loading, transfer, elution, and equilibration. At the loading step, the prepared urine or hemodialysate sample is directly injected onto the extraction column, where the matrix components are rapidly washed away and the analytes are retained with mobile phase A at a high flow rate (5 ml/min). At the transfer step, the analytes are eluted from the extraction column and transferred onto a reversed-phase analytical column at a flow rate of 0.2 ml/min with a mobile phase that is stored in the transfer loop and filled before the loading step. At the same time, a mobile phase composed of 100% A is delivered at a flow rate of 0.25 ml/min from the eluting pump, and it is mixed with the mobile phase that is transferred from the extraction column and just before the analytical column allowing the analytes to focus onto the analytical column. At the elution step, the analytes are separated on the analytical column and eluted to the mass spectrometer with the mobile phase composed of 70% and 85% of B for the urine and hemodialysate assay, respectively; then the extraction column is washed to reduce the carryover. At the equilibration step, the loop is filled with the mobile phase composed of 30% and 40% of B for the urine and hemodialysate assay, respectively; and then both the extraction and analytical columns are equilibrated with 100% A for the next injection.

2.6. Mass spectrometry conditions

Precursor ions for sitagliptin and internal standard were determined from Q1 spectra obtained during infusion of neat solution of each compound, via the turbo ion spray (TIS) source into the mass spectrometer operated in positive ionization mode with the collision gas off. Under these conditions, the analyte and internal standard yielded predominantly protonated molecules at *m/z* 408 and *m/z* 422. Each of the precursor ions was subjected to collision-induced dissociation (CID) in order to generate product ions. The product ion of sitagliptin at *m/z* 235 and internal standard at *m/z* 249 were chosen for the selected reaction monitoring (SRM) (Fig. 2). Experiment parameters were optimized during the infusion of sitagliptin through the TIS interface. For hemodialysate assay (API4000),

Table 3
Sitagliptin concentrations (μg/ml) in human urine samples collected from a single oral dose study

Dose (mg)	Time (h)							
	Predose	0–2	2–4	4–8	8–12	12–24	24–36	36–48
25 fed	BLOQ[a]	0.255	16.4	15.3	13.5	4.20		
	BLOQ	7.88	7.19	18.8	2.31	3.42		
	BLOQ	5.97	33.7	8.14	2.53	3.53		
	BLOQ	2.76	4.35	2.97	1.73	1.32		
	BLOQ	13.9	4.88	3.63	7.41	7.18		
	BLOQ	3.48	7.05	4.13	2.42	2.87		
25	BLOQ	4.42	10.1	37.4	4.88	6.30		
	BLOQ	2.15	3.63	8.85	7.28	6.22		
	BLOQ	5.56	28.7	4.51	3.26	4.84		
	BLOQ	4.12	4.15	2.93	1.66	1.34		
	BLOQ	1.67	5.60	5.26	5.12	9.25		
	BLOQ	6.45	4.09	6.89	4.64	6.07		
50	BLOQ	3.85	11.1	14.7	9.88	9.74		
	BLOQ	171	168	39.3	17.8	17.1		
	BLOQ	11.3	20.5	14.9	15.0	27.2		
	BLOQ	3.95	16.9	17.8	15.7	20.5		
	BLOQ	8.41	43.6	11.6	7.20	14.8		
	BLOQ	3.04	27.5	20.5	21.7	18.2		
100	BLOQ	4.91	22.5	12.0	5.15	8.70	9.78	2.25
	BLOQ	12.5	55.5	101	19.4	24.5	9.86	3.64
	BLOQ	40.0	156	18.8	12.6	20.7	6.08	3.17
	BLOQ	13.5	14.4	16.2	8.16	6.09	1.64	0.700
	BLOQ	8.47	33.8	25.2	15.5	11.8	8.15	0.659
	BLOQ	16.5	37.7	34.2	17.1	35.2	7.01	4.22
200	BLOQ	680	595	313	164	61.1	24.7	8.37
	BLOQ	108	223	103	128	62.0	14.0	8.81
	BLOQ	205	320	95.5	149	126	68.0	12.8
	BLOQ	47.1	88.1	58.4	140	82.5	18.4	8.72
	BLOQ	149	237	204	157	63.0	26.4	7.61
	BLOQ	39.9	117	115	170	76.6	15.9	8.24

[a] Below the lower limit of quantitation (0.100 μg/ml).

Table 4
Intraday precision and accuracy for the determination of sitagliptin in five lots of human urine using HTLC–MS/MS

Nominal concentration (μg/ml)	Mean found concentration[a] (μg/ml, $n=5$)	Precision R.S.D.% ($n=5$)	Accuracy[b] (%)
0.100	0.100	6.3	100
0.200	0.194	5.3	97.1
0.500	0.528	4.2	106
2.00	1.96	2.5	97.9
5.00	5.22	2.3	104
20.0	20.0	2.9	100
40.0	38.8	6.5	96.9
50.0	48.7	5.0	97.4

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve using all five replicates at each concentration.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.

the ionspray voltage was 3000 V and the TIS interface temperature was maintained at 700 °C. Nitrogen was used as nebulizer, curtain and collision gas. The declustering potential was 52 V, entrance potential was 8 V, collision energy was 26 V, and collision cell exit potential was 12 V. For urine assay (API365), the ionspray voltage was 4000 V and the TIS interface temperature was maintained at 480 °C. Nitrogen was used as nebulizer, curtain and collision gas. The declustering potential was 15 V, entrance potential was 8.6 V, collision energy was 26 V, and collision cell exit potential was 11 V. Instrument settings were adjusted to maximize the response for the sitagliptin precursor/product ion transitions of *m/z* 408 → 235.

3. Results and discussions

3.1. Method modification

Initially, the sitagliptin HTLC/LC–MS/MS urine assay was validated based on the human plasma assay protocol [2]. However, due to higher concentration of sitagliptin in the urine samples than that in plasma, urine samples had to be diluted about 100–200-folds before analysis (Table 3). In order to reduce the number of samples with dilution, the method was modified to have the relevant LLOQ and calibration range for the clinical studies. A less sensitive mass spectrometer, API 365, was used for urine assay (versus API 4000 which was needed to achieve the requisite sensitivity for the plasma assay). In addition, two of the mobile phases used in the plasma assay [2], 15% acetic acid and 90% THF, for removing proteins and lipids in human plasma samples, were eliminated. The detailed procedure of the HTLC/LC online extraction method is shown in Table 1. The modified method was validated and met the FDA guidance requirements [20]. The LLOQ of the urine assay was 0.1 μg/ml and the calibration range of the assay was from 0.1 to 50 μg/ml.

Based on the requirement of the pharmacokinetic study, the expected LLOQ for the hemodialysate assay was 0.01 ng/ml which was 50 times lower than the LLOQ of the plasma assay. Theoretically, increasing the injection volume and reducing the dilution factor during sample preparation was the easiest way to improve the LLOQ of the assay. However, this approach alone did not achieve the projected LLOQ. Therefore, the HTLC/LC method was modified to further increase the recovery of the sitagliptin on the extraction column and the ionization efficiency in the source (Table 2). To increase the recovery of sitagliptin on the extraction column, the loading time was reduced from 30 to 24 s because the hemodialysate sample contains much less protein than the plasma sample. To increase the ionization efficiency of sitagliptin, an 85% B mobile phase was used in the eluting step compared to the 40–70% B grading for the plasma assay. The use of the 85% B mobile phase in the eluting step not only increased the ionization efficiency, but also improved the peak

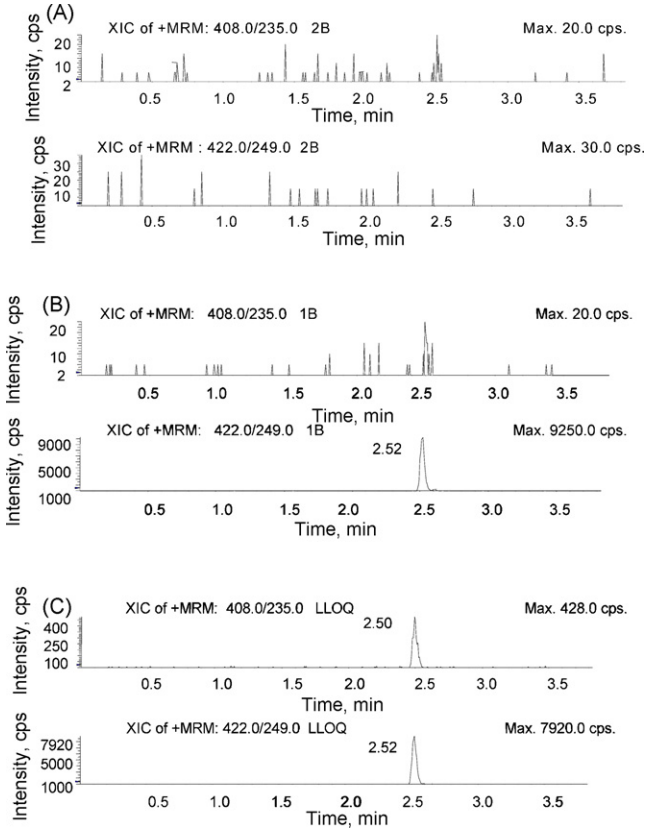

Fig. 3. Chromatograms of the urine double blank (A), single blank (B) and 0.1 μg/ml standard sample (C). Experimental conditions: Cohesive 2300 HTLC turboflow system; extraction column, Cyclone HTLC (50 mm × 1.0 mm, 60 μm); analytical column, BDS Hypersil C18 (30 mm × 2.1 mm, 3 μm). Mobile phase A: 2.5 mM ethylamine, 0.1% formic acid in water; mobile phase B: 0.1% FA in acetonitrile.

shape of sitagliptin. The peak width at half height was 2 s compared with 3 s of the plasma and urine assays. Based on these modifications, the sensitivity of the hemodialysate assay was significantly increased. The LLOQ of the hemodialysate assay was 0.01 ng/ml when 40 μl of prepared hemodialysate sample was injected.

3.2. Effect of reagents on the selectivity

To control the pH of samples, 20% and 5% acetic acid were added to plasma and urine samples during the sample preparation, respectively. The final concentration of acetic acid in the prepared sample was 2.5% for the plasma assay and 0.7% for the urine assay. HPLC grade acetic acid was used for both plasma and urine assay, and no interfering components were found. However, when 5% acetic acid was added in the hemodialysate samples, a peak, which was similar to the peak height of LLOQ at the retention time of sitagliptin, was found in all six different lots of the control hemodialysate samples (double blank). Further investigation demonstrated that the interfering component was introduced from the acetic acid. When acetic acid was replaced by formic acid during the sample preparation, the interfering peak disappeared. The occurrence of an interfering component was very likely due to the very high sensitivity of the hemodialysate assay.

3.3. Specificity

To demonstrate that no interfering components elute at the retention times of sitagliptin or internal standard, the specificity of both assays was evaluated by testing six different lots of control urine and hemodialysate. No endogenous interferences were found at the retention times of sitagliptin or internal standard for both assays (Figs. 3 and 4).

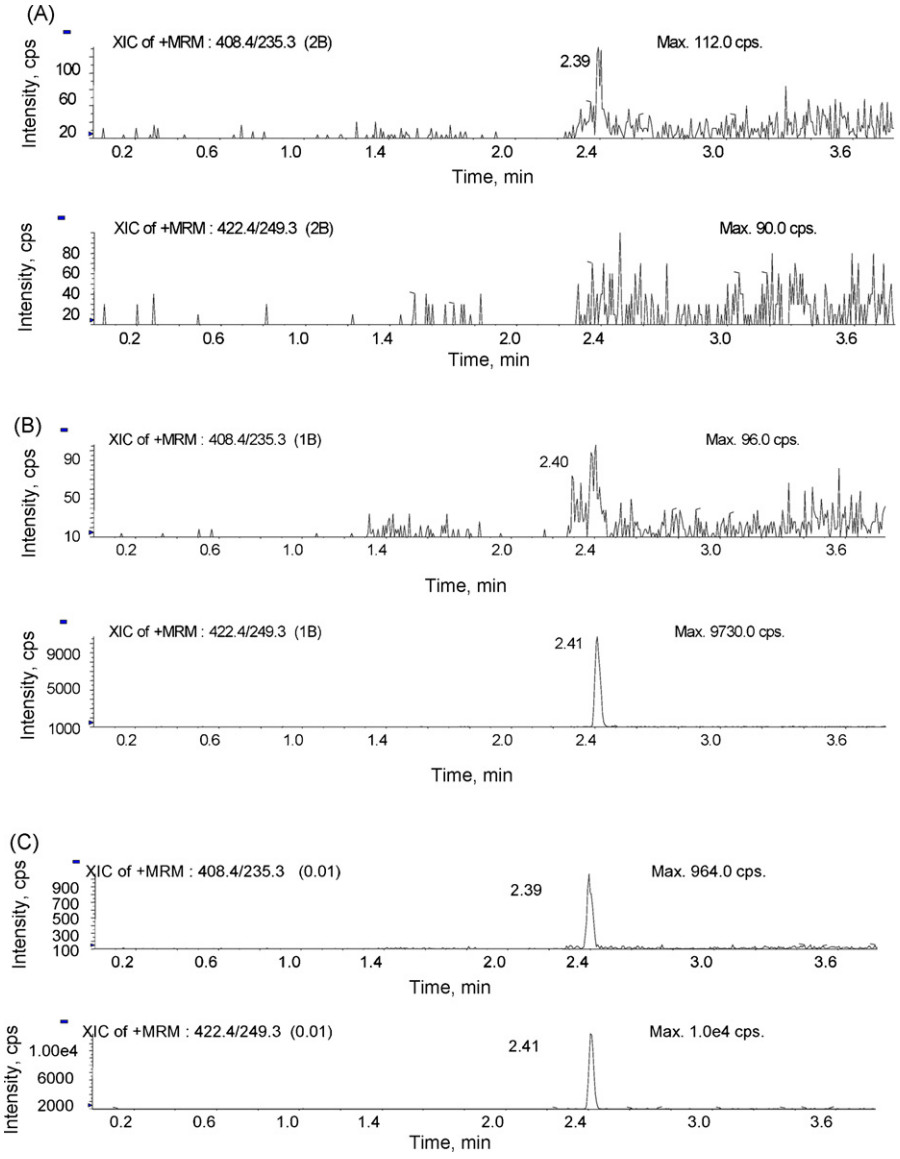

Fig. 4. Chromatograms of the hemodialysate double blank (A), single blank (B) and 0.01 ng/ml standard sample (C). Experimental conditions: same as in Fig. 3.

Table 5
Intraday precision and accuracy for the determination of sitagliptin in five lots of hemodialysate using HTLC–MS/MS

Nominal concentration (ng/ml)	Mean found concentration[a] (ng/ml, $n=5$)	Precision R.S.D.% ($n=5$)	Accuracy[b] (%)
0.0100	0.0100	3.2	100
0.0200	0.0207	4.1	103
0.0500	0.0449	2.7	89.8
0.200	0.202	2.8	101
0.500	0.493	2.6	98.6
2.50	2.52	4.1	101
4.00	4.15	3.9	104
5.00	5.13	1.6	102

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve using all five replicates at each concentration.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.

Table 6
Intraday and interday precision and accuracy of QC samples for the determination of sitagliptin in human urine

	Nominal concentration (ng/ml)	Mean found concentration[a] (ng/ml)	Precision R.S.D.%	Accuracy[b] (%)
Intraday $n=5$	0.300	0.289	1.8	96.2
	5.00	5.30	1.9	106
	40.0	41.1	2.6	103
Interday[c] $n=52$	0.300	0.314	5.5	105
	5.00	5.26	5.2	105
	40.0	40.6	3.8	102

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.
[c] Number of analytical runs = 18.

3.4. Matrix effect

The absence of a relative matrix effect on ionization (suppression or enhancement) for both assays were demonstrated by determining intraday precision and accuracy in five different lots of urine and hemodialysate. The intraday precision and accuracy values obtained in five different lots of urine and hemodialysate meet the criteria described in FDA's guidance [20]. Therefore, relative matrix effects were considered as not having a significant impact on assay performance (Tables 4 and 5).

3.5. Sensitivity and calibration range

For sitagliptin calibration range, the urine assay was validated from 0.1 to 50 μg/ml and the hemodialysate assay was validated from 0.01 to 5 ng/ml. Weighted ($1/x$ and $1/x^2$ for urine and hemodialysate, respectively) least-squares linear regression calibration curves were constructed by plotting the peak area ratios of analyte to internal standard versus standard concentrations. All samples with concentrations above the highest standard were reanalyzed with the validated dilution procedure.

3.6. Accuracy and precision

Intraday accuracy and precision of the methods were evaluated by analyzing replicate calibration standards ($n=5$) at all concentrations used to construct the standard curves. These five sets of calibration standards were prepared from five different lots of human control urine or hemodialysate. For the urine assay, the precision and accuracy were from 2.3% to 6.5% (R.S.D.%) and 96.9% to 106% of nominal value, respectively. For the hemodialysate assay, the precision and accuracy were from 1.6% to 4.1% (R.S.D.%) and 89.8% to 104% of nominal value, respectively. Data are shown in Tables 4 and 5 for urine and hemodialysate assay, respectively.

Low, medium and high concentration QC samples containing sitagliptin were prepared at concentrations of 0.3, 5 and 40 μg/ml for the urine assay and concentrations of 0.03, 0.5 and 4 ng/ml for the hemodialysate assay. The intraday precision (R.S.D.%, $n=5$) for urine QC samples varied from 1.8% to 2.6% and the accuracy from 96.2% to 106% of nominal value. The intraday precision for hemodialysate QC samples varied from

Table 7
Intraday precision and accuracy of QC samples for the determination of sitagliptin in hemodialysate

Nominal concentration (ng/ml)	Mean Found concentration[a] (ng/ml, $n=5$)	Precision R.S.D.% ($n=5$)	Accuracy[b] (%)
0.0300	0.0332	8.9	111
0.500	0.504	2.3	101
4.00	3.99	3.5	99.8

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.

Table 8
Freeze-thaw and room temperature stability of QC samples in human urine

	Nominal concentration (ng/ml)	Mean found concentration[a] (ng/ml)	Precision R.S.D.%	Accuracy[b] (%)
Freeze-thaw stability 3 cycles, $n=3$	0.300	0.298	3.2	99.3
	5.00	4.98	0.12	99.7
	40.0	40.8	0.98	102
Room temperature stability 6 h, $n=3$	0.300	0.299	3.2	99.6
	5.00	5.25	0.40	105
	40.0	41.5	3.5	104

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.

Table 9
Freeze-thaw and room temperature stability of QC samples in hemodialysate

	Nominal concentration (ng/ml)	Mean found concentration[a] (ng/ml)	Precision R.S.D.%	Accuracy[b] (%)
Freeze-thaw stability 3 cycles, $n=3$	0.0300	0.0301	2.1	100
	0.500	0.506	2.0	101
	4.00	3.99	3.6	99.8
Room temperature stability 6 h, $n=3$	0.0300	0.0312	1.6	104
	0.500	0.494	2.6	98.8
	4.00	4.09	0.61	102

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.

2.3% to 8.9% and the accuracy from 99.8% to 111% of nominal value (Tables 6 and 7).

The interday variability of the assays was evaluated by analyzing QC samples daily with clinical samples. For the urine assay, the interday precision and accuracy shown in Table 5 represents 52 sets of low, medium and high concentration QC samples over a 6-month period for two analysts. The precision and accuracy varied from 3.8% to 5.5% and 102% to 105%, respectively.

3.7. Stability

Freeze-thaw stability of sitagliptin in urine and hemodialysate was investigated by examining the stability of QC samples over three freeze-thaw cycles. Three sets of low, medium and high urine and hemodialysate QC samples were thawed at room temperature for more than 2 h and then frozen at −20 °C overnight for each cycle. No significant concentration differences were observed for the urine or hemodialysate QC samples subjected to three freeze-thaw cycles compared to the nominal concentration (Tables 8 and 9).

Room temperature storage stability was studied by examining urine and hemodialysate QC samples exposed to ambient temperature for a period of 6 h before analysis. Three sets of low, medium and high urine QC samples (0.3, 5 and 40 μg/ml) and hemodialysate QC samples (0.03, 0.5 and 4 ng/ml) were prepared and analyzed. For both urine and hemodialysate QC samples, no significant concentration differences were observed for each concentration compared with nominal concentration (Tables 8 and 9).

Autosampler storage stability of the processed samples was evaluated for both assays. Five sets of low, medium and high concentration QC samples were processed and analyzed with a set of calibration standards, and the concentrations of sitagliptin in the QC samples were calculated. The next day, a fresh standard curve was prepared and analyzed alones with the processed

Table 10
Autosampler stability of QC samples in human urine and hemodialysate

	Nominal concentration[a]	Initial found concentration[a]	Accuracy[b] (%)	Found concentration[a] after storage	Accuracy[b] after storage (%)
Urine (μg/ml) 5 °C, 27 h, $n=5$	0.3	0.289	96.3	0.321	107
	5	5.30	106	5.06	101
	40	41.1	103	40.8	102
Hemodialysate (ng/ml) 5 °C, 20 h, $n=5$	0.03	0.0332	111	0.0301	100
	0.5	0.504	101	0.476	95.2
	4	3.99	99.8	3.83	95.8

[a] Mean found concentrations calculated from the weighted linear least-squares regression curve.
[b] Expressed as (mean found concentration/nominal concentration) × 100%.

QC samples of the previous days, which had been stored in the 5 °C autosampler overnight. The concentrations of the sitagliptin QC samples were calculated based on the newly prepared standard curve. The concentrations of low, medium and high hemodialysate QC samples were 0.0332, 0.504 and 3.99 ng/ml for the initial analysis, and 0.0301, 0.476 and 3.83 ng/ml after storage at 5 °C for 20 h. The concentrations of sitagliptin in urine QC samples were 0.289, 5.30 and 41.1 μg/ml for the initial analysis and 0.321, 5.06 and 40.8 μg/ml after storage at 5 °C for 27 h. No significant concentration differences were observed for both assays (Table 10).

4. Conclusions

To meet the assay sensitivity requirement of the clinical studies, two simple and robust turbulent flow online extraction HTLC/LC–MS/MS methods for quantifying sitagliptin in urine and hemodialysate were developed based on the sitagliptin plasma assay [2]. The LLOQ and calibration range of hemodialysate assay were 0.01 ng/ml and 0.01–5.0 ng/ml, respectively. The sensitivity of hemodialysate assay was increased 50-fold compared with plasma assay. The LLOQ and calibration range of the urine assay were 0.1 μg/ml and 0.1–50 μg/ml, respectively. The validation and interday QC results demonstrated that both methods are selective, accurate, precise, reproducible, and suitable for quantifying sitagliptin in human urine and hemodialysate samples.

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