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Determination of sitagliptinin human plasma using protein precipitation and tandem mass spectrometry

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ABSTRACT

A simple offline LC–MS/MS method for the quantification of sitagliptin in human plasma is described. Samples are prepared using protein precipitation. Filtration of the supernatants through a Hybrid-SPE-PPT plate was found to be necessary to reduce ionization suppression caused by co-elution of phospholipids with sitagliptin. The sitagliptin and its stable isotope labeled internal standard (IS) were chromatographed under hydrophilic interaction chromatography conditions on a Waters Atlantis HILIC Silica column (2.1 mm × 50 mm, 3 μm) using a mobile phase of ACN/H_2O (80/20, v/v) containing 10 mM NH_4Ac (pH 4.7). The sample drying after protein precipitation due to high organic content in the sample is not necessary, because HILIC column was used. The analytes were detected with a tandem mass spectrometer employing a turbo ion spray (TIS) interface in positive ionization mode. The multiple reaction monitoring (MRM) transitions were m/z 408 → 235 for sitagliptin and m/z 412 → 239 for IS. The lower limit of quantitation (LLOQ) for this method is 1 ng/mL when 100 μL of plasma is processed. The linear calibration range is 1–1000 ng/mL for sitagliptin. Intra-day precision and accuracy were assessed based on the analysis of six sets of calibration standards prepared in six lots of human control plasma. Intra-day precision (RSD%, $n = 6$) ranged from 1.2% to 6.1% and the intra-day accuracy ranged from 97.6% to 103% of nominal values.

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1. Introduction

Sitagliptin, (2R)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]butan-2-amine (Fig. 1), is an orally active, potent and selective inhibitor of dipeptidyl peptidase-IV (DPP-IV), which has been marketed in USA, Europe and other countries for the treatment of type 2 diabetes. DPP-IV inhibitors enhance levels of active glucagon-like peptide 1 (GLP-1) and other incretins, and facilitate glucose-dependent insulin secretion [1,2]. In addition, GLP-1 inhibits glucagon release, slows gastric emptying, reduces appetite, and regulates the growth and differentiation of the insulin producing β cells in pancreatic islets.

To support sitagliptin clinical studies, several analytical methods were developed and validated using high turbulence liquid chromatography (HTLC) online extraction with tandem mass spectrometry (MS/MS) for the quantification of sitagliptin in human plasma, urine and dialysate [3,4]. These methods were simple and rugged, and have been used to support many clinical studies. However, since the online extraction required a high turbulence liquid chromatography (HTLC) system, method transfer between laboratories was found to be challenging in terms of personal training and instrumentation adjustment [5].

An offline method for the quantification of sitagliptin in human plasma was reported recently [6]. The method was based on a liquid–liquid extraction of sitagliptin from human plasma followed by LC–MS/MS analysis. The method utilizes a relatively large (500 μL) sample volume and a manual, low-throughput sample preparation procedure (i.e., liquid/liquid extraction in individual tubes). Moreover, the retention time (0.94 min) of sitagliptin and IS suggests that the analytes eluted close to the column void volume (k' close to 0) under the reported chromatographic conditions, indicating the potential for significant matrix effects on ionization, which might not be fully compensated by the non-structurally related internal standard employed by this method.

The purpose of the research described herein was to develop and validate a simple, high-throughput LC–MS/MS method for the quantification of sitagliptin in human plasma. The assay utilizes protein precipitation/filtration for sample preparation prior to LC–MS/MS analysis. The use of a filter plate to eliminate the phospholipids from prepared samples to reduce the matrix effects is also discussed. Results of assay validation, including assessment of intra-day and inter-day precision and accuracy, quality control sample (QCs) stability, incurred sample reproducibility, and potential interference from concomitant medicines are presented.

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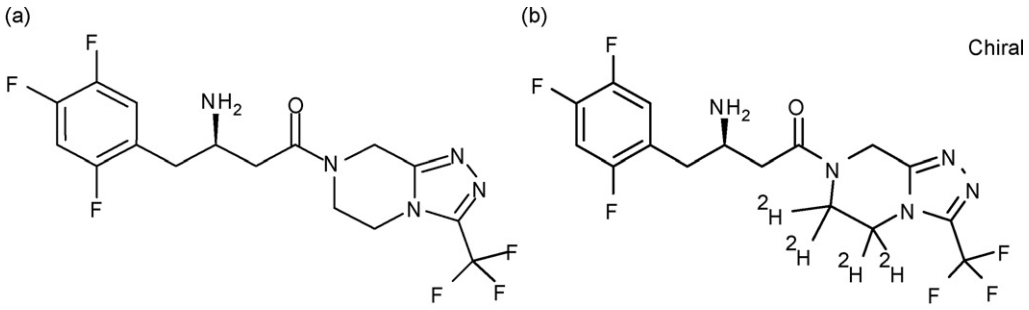

Fig. 1. Chemical structures of sitagliptin (a) and stable isotope labeled sitagliptin (b).

2. Experimental

2.1. Materials

Sitagliptin (Fig. 1) was obtained from Merck Research Laboratories (Rahway, NJ, USA) and stable isotope labeled sitagliptin (Fig. 1) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). The purity of sitagliptin and stable isotope labeled sitagliptin was 100% and 98%, respectively. The isotopic purity of stable isotope labeled sitagliptin was 99%, Drugs evaluated for assay interference were purchased from several different companies. Pioglitazone, simvastatin and simvastatin lactone were obtained from Merck Research Laboratories (Rahway, NJ, USA). Metformin was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Atorvastatin, atorvastatin lactone, ortho-hydroxy atorvastatin, ortho-hydroxy atorvastatin lactone, para-hydroxy atorvastatin, para-hydroxy atorvastatin lactone and M-II hydroxy pioglitazone were purchased from Synfine Research, Inc. (Richmond Hill, ON, Canada). Acetaminophen, caffeine, ibuprofen, pheniramine, dextromethorphan (DXM), nicotine, pseudoephedrine and diphenhydramine were purchased from Cerilliant (Round Rock, TX, USA). Acetylsalicylic acid was purchased from Acros Organics (Morris Plains, NJ, USA). Phenylephrine was purchased from USP (Rockville, MD, USA). HPLC grade acetonitrile (ACN), tetrahydrofuran (THF), ammonium acetate (NH_4Ac) and formic acid (98–100%) were purchased from Sigma–Adrich (St. Louis, MO, USA). Control human plasma with K_2 EDTA as the anticoagulant was purchased from Biological Specialty Corp. (Colmar, PA, USA). Hybrid-SPE-PPT 96-well plate was purchased from Sigma–Adrich (St. Louis, MO, USA).

2.2. Instruments

A Shimadzu LC-10ADVP HPLC pump controlled by Cohesive Aria™ software (Cohesive Technologies, Inc., MA, USA) was used for analysis. A CTC HTS PAL autosampler (CTC Analytics, Zingen, Switzerland) was used for sample injection. A Sciex API 4000 mass spectrometer (Toronto, Canada) was used as the detector. A Packard MultiPROBE II HT EX robotic liquid handling system (Meriden, CT, USA) was used in all pipetting steps for sample preparation. Peak areas generated using SCIEX Analyst® software package (Sciex, Toronto, Canada) are exported into the WATSON system (v 7.3) for quantitation.

2.3. Standard solutions and quality control (QC) samples

The stock solutions of sitagliptin and stable isotope labeled sitagliptin were prepared at a concentration of 1.00 mg/mL in 50% ACN (ACN/water, 50/50, v/v). Working standard solutions from 2.00 to 2000 ng/mL (2.00, 4.00, 10.0, 40.0, 200, 1000, 1600 and 2000 ng/mL) in 80% ACN (ACN/water, 80/20, v/v) were prepared by serial dilution from the stock solution of sitagliptin. The working internal standard (IS) solution was prepared at a concentration of 200 ng/mL in 80% ACN by dilution from the stock solution of stable isotope labeled sitagliptin.

A separate weighing was used to prepare a quality control (QC) stock solution of sitagliptin at a concentration of 5.00 mg/mL in 50% ACN. The QC working solutions (0.30, 10 and 80 μg/mL) in 50% ACN were prepared by serial dilution from QC stock solution of sitagliptin. QC samples were prepared by adding appropriate volumes of QC working solutions into volumetric flasks and diluting to volume with control human plasma to achieve the desired concentrations of 3.00, 100 and 800 ng/mL for low, middle and high QCs, respectively. Aliquots (500 μL) of each QC sample were transferred into Nunc Cryo vials, which were capped and stored at −20 °C.

QC samples were processed along with the clinical samples during each analytical run. The minimum number of QC samples was at least 5% of the number of unknown samples analyzed in a given run or six total QCs, whichever was greater [7].

2.4. Preparation of plasma standards, QCs and clinical samples

Plasma standards were prepared by mixing 50 μL of each working standard with 100 μL aliquots of control human plasma that were pipetted into individual wells of a 96-well plate. Aliquots (100 μL) of QC and subject samples were pipetted into individual wells of the assay plate. A 50 μL aliquot of make-up solvent (80% ACN in water) was added to the wells containing the QC and subject samples. IS working solution (50 μL) was then added to each well. The contents of the wells were mixed, after which 600 μL of ACN with 0.1% FA was added to each sample to precipitate the protein. Following centrifugation, the supernatants were filtered through a 96-well Hybrid-SPE-PPT plate. The filtered samples were then further diluted using ACN with 0.1% FA such that the final acetonitrile content of the samples was approximately 92%.

2.5. Chromatographic conditions

A solution of ACN/H_2O (80/20, v/v) containing 10 mM NH_4Ac (pH 4.7) was used as mobile phase for isocratic elution, and a Waters Atlantis HILIC column (2.1 mm × 50 mm, 3 μm) was used at room temperature as analytical column. The flow rate of the mobile phase was 0.3 mL/min. Under these conditions, the retention times for both sitagliptin and stable isotope labeled sitagliptin were about 1.05 min; the total run time was 4.0 min.

2.6. Mass spectrometry detection conditions

Precursor ions for sitagliptin and stable isotope labeled sitagliptin were determined from the Q1 positive ion spectra that were obtained during the infusion of a neat solution of each compound, via the TurboIonSpray™ (TIS) source, into the mass

spectrometer. Under these conditions, the analyte and internal standard yielded predominately the protonated molecular ions of m/z 408 and m/z 412. Each of the precursor ions was subjected to collision induced dissociation (CID) in order to generate product ions. Sitagliptin gave rise to a predominant product ion of m/z 235 and the stable isotope labeled sitagliptin gave the corresponding product ion of m/z 239. Based on these spectra, the ion transitions of m/z 408 → 235 (sitagliptin) and m/z 412 → 239 (IS) were used for MRM. MS parameters were further optimized by infusing a solution of sitagliptin through the TIS interface. The ion spray voltage was optimized at 4500 V and the TIS interface temperature was maintained at 550 °C. Nitrogen was used as nebulizer, curtain and collision gas, respectively. The declustering potential was 70 V, entrance potential was 9 V, collision energy was 27 V, and collision cell exit potential was 16 V.

3. Results and discussion

3.1. Chromatography

Sitagliptin is a relatively polar compound that is highly soluble in water. Our initial attempt to use reversed-phase HPLC conditions for the determination of sitagliptin was not successful. Specifically, even when the organic content in the mobile phase was reduced to 50%, sitagliptin was not retained on any of the following columns: Waters Atlantis T3, Waters dC18, Waters XBridge RP C18, BDS Hypersil C18 or Hypersil GOLD. Using mobile phases containing less organic solvent was undesirable in that analyte ionization efficiency, and hence, assay sensitivity was adversely affected.

Hydrophilic interaction chromatography (HILIC) is an alternative chromatographic technique that offers complementary selectivity to reversed-phase chromatography (RP). It has been widely used for retaining very polar organic molecules that may not be retained by RP columns [8]. Unlike the highly aqueous mobile phases required for polar molecule retention in RP separations, HILIC conditions employ high percentages of organic solvent in the mobile phase, which is ideal for optimizing mass spectrometric sensitivity [9].

The Atlantis HILIC Silica column (2.1 mm × 50 mm, 3 μm) was evaluated with acetonitrile (ACN) as mobile phase and 10 mM NH_4Ac (pH 4.7) as additive. The buffer pH and ACN content in mobile phase were selected based on the retention and peak shape. The mobile phase pH was tested under 3.0, 4.7 and 7.0, and pH 4.7 presented the best retention. When the ACN content was less than 75% in the mobile phase, the peak shape of sitagliptin was sharp and symmetrical, but the k' of sitagliptin was less than 1.0. Conversely, when the ACN content was greater than 85%, the k' of sitagliptin was greater than 2.0, however, the shape of sitagliptin peak started to broaden. At 80% ACN, the peak shape of sitagliptin was reasonably sharp and the k' of sitagliptin was near to 2.0, hence a mobile phase of 80/20 (v/v%) ACN:water containing 10 mM NH_4Ac (apparent pH of 4.7) was selected as the mobile phase for the quantification of sitagliptin in human plasma.

Injection solvent strength is known to affect chromatographic peak shape [10,11]. For reversed-phase chromatography, the organic content in the processed sample mix should be equal to or lower than that of mobile phase [10]. It is suggested that sample solvent strength should be no more than half the mobile phase strength [11]. Similarly, under HILIC conditions, the sample solvent strength should also be weaker than the mobile phase [12]. Because ACN is the weak solvent for HILIC, the ACN content in the sample needed to be higher than 80% for sitagliptin determination. To evaluate the influence of sample solvent strength on analyte peak shape, two processed plasma samples containing 85% and 92.5% ACN, respectively, were prepared and assayed on several different lots of new and used HPLC columns. The peak shape for the sample containing 92.5% ACN was symmetrical and sharp on all new and used columns. However, the peak shapes for the sample containing 85% ACN were not consistent; broadened, and occasionally split, peaks were observed. Based on these observations, it was concluded that higher acetonitrile content in the injection solvent led to less variable, and hence, more rugged, chromatographic results. For the sitagliptin determination, ACN content in the prepared sample was 85% after adding 600 μL of ACN to precipitate the proteins in the plasma. To further increase ACN content in the prepared samples, the filtered supernatant was diluted with 100% ACN to further increase the ACN content to about 92% in the final sample.

In a similar manner, autosampler wash solvents should be carefully selected and evaluated during the method development because wash solvents are not only used to eliminate carryover, but may also, because of residual solvent remaining in the system, affect analyte peak shape. For the sitagliptin determination, a 50/50 (v/v%) ACN:water mixture containing 0.1% FA was used as the first needle wash solvent to eliminate analyte carryover, and a weak wash solution consisting of 95% ACN was used as the second needle wash to flush the system prior to sample aspiration. Occasional peak splitting was observed if only the first needle wash solvent was used.

3.2. Matrix effect and recovery

Mass spectrometry as an HPLC detector has been widely employed in different research areas because of its sensitivity, selectivity and specificity. However, matrix effects, a phenomenon of ion suppression or enhancement of the analyte of interest, needs to be evaluated during method development because the assay accuracy and precision of a LC–MS/MS method could be significantly affected by the phenomena [13,14]. Endogenous matrix components co-eluting with the analyte of interest are believed to be the primary cause of ionization matrix effects.

The absolute matrix effect may be quantitatively assessed by comparing the response of the analyte spiked into extracted blank matrix with the response of the analyte spiked into matrix-free reconstitution solution [13]. Using this method, the matrix effect for the determination of sitagliptin was evaluated, and the initial results showed that absolute matrix effects ranged from 119% to 201%, at the tested concentrations (Table 1).

Plasma phospholipids have been identified as a major contributing source of matrix effects in LC–MS/MS based bioanalytical methods [15–17]. Phosphatidylcholines (PC), sphingomyelins (SM) and lysophosphatidylcholines (lyso-PC) represent about 80–90% of total plasma phospholipids [18]. These endogenous substances may be detected by using a positive precursor ion scan of m/z 184 [19]. This approach was used to monitor phospholipids in plasma extracts after protein precipitation. The results showed that there was a cluster of components co-eluting with sitagliptin (Fig. 2a), suggesting the potential for phospholipid-based matrix effects.

Since attempts to change chromatographic conditions to separate the analyte from interference components were not successful, our attention turned toward methodology to eliminate the presence of these components during sample preparation. Recently, a filtration plate containing a zirconia based sorbent (Hybrid-SPE) has become commercially available from Supelco, Sigma–Aldrich. Use of this sorbent has been reported to be effective for the removal of phospholipids from plasma samples [20], thus this approach was evaluated for application to the sitagliptin assay.

Our results showed that filtration through the Hybrid-SPE-PPT plate after protein precipitation dramatically reduced the level of phospholipids in plasma samples (Fig. 3b), and lead to a significant reduction in absolute matrix effects (Table 1).

Table 1
Absolute matrix effect of sitagliptin and stable isotope labeled sitagliptin in protein precipitated human plasma before and after filtration through Hybrid-SPE-PPT plate with the corresponding recovery.

Standard concentration in plasma (ng/mL)	Mean absolute matrix effect[a] before filtration (%, $n=6$)	Mean absolute matrix effect[a] after filtration (%, $n=6$)	Mean recovery[b] after filtration (%, $n=6$)
1.00	201	90.7	100
100	119	82.7	99.4
1000	119	87.6	98.8
ISTD[c]	122	85.2	98.9

[a] Expressed as (the mean peak area of analyte spiked after extraction/the mean peak area of the neat analyte standard) × 100%.
[b] Expressed as (the mean peak area of analyte spiked before extraction/the mean peak area of analyte spiked after extraction) × 100%.
[c] $n=18$.

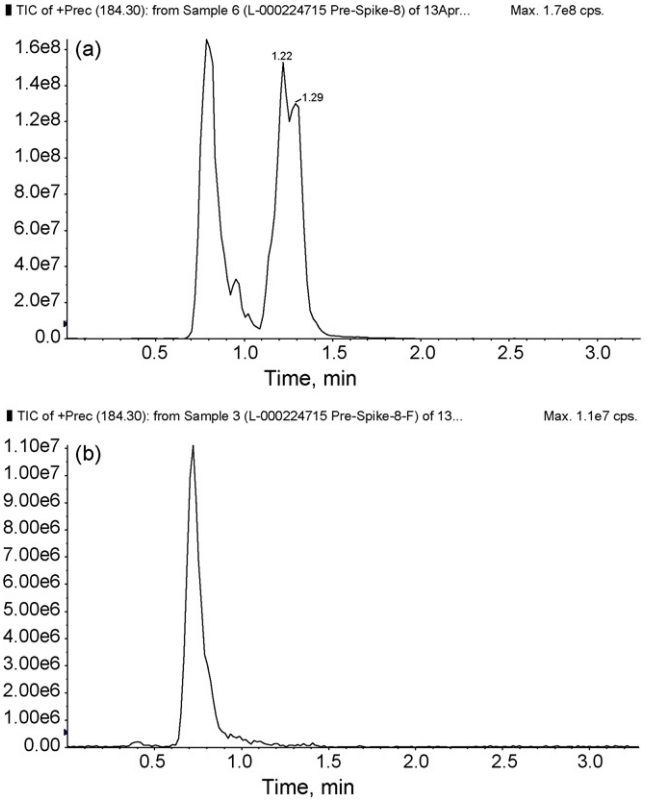

Fig. 2. Positive ESI precursor ion scan of m/z 184 for protein precipitated plasma sample before (a) and after (b) filtered through Hybrid-SPE-PPT plate.

The recovery could be determined by comparing the response of the analyte spiked into matrix with the response of the analyte spiked into extracted blank matrix [13]. The recovery of sitagliptin and its IS utilizing protein precipitation/filtration for sample preparation was near 100% (Table 1).

3.3. Assay selectivity

The assay selectivity was assessed using samples from six lots of human control plasma. No interferences were observed at the retention times of sitagliptin and its internal standard (Fig. 3a) in any of these lots.

The absence of "cross-talk" between channels used for monitoring the analytes was confirmed by the analysis of plasma samples containing internal standard at the working concentration in the absence of sitagliptin and the analysis of plasma samples containing sitagliptin at 1000 ng/mL in the absence of internal standard (Fig. 3b and c). No "cross-talk" was observed in either experiment.

3.4. Accuracy and precision

Weighted ($1/x^2$) least-squares regression calibration curves, constructed by plotting the peak area ratios of analyte to internal standard versus concentrations of calibration standards were linear over the concentration range of 1.00–1000 ng/mL. The lower limit of quantification (LLOQ) was 1.00 ng/mL.

Intra-day accuracy and precision of the method was evaluated by analyzing six sets of calibration standard curves prepared in six different lots of human control plasma (Table 2). The precision (RSD%, $n=6$) ranged from 1.2% to 6.1% and the accuracy from 97.6% to 103% of the nominal values. The intra-day data demonstrated the relative matrix effects did not have impact on the assay precision and accuracy.

Low, middle and high QC samples containing sitagliptin were prepared at concentrations of 3.00, 100 and 800 ng/mL in human plasma. The intra-day precision (RSD%, $n=5$) for QCs varied from 0.67% to 1.2% and accuracy from 97.0% to 104% of the nominal value (Table 3).

Precision and accuracy at the LLOQ were evaluated by analyzing five freshly prepared LLOQ samples along with a calibration curve. The intra-day precision (RSD%, $n=5$) at the LLOQ varied from 4.8% to 5.0% and accuracy from 94.4% to 99.0% of the nominal values (Table 4). The inter-day precision (RSD%, $n=3$) at the LLOQ obtained from three independent runs on three days was 2.8% and accuracy was 96.0% of the nominal value (Table 4).

Table 2
Intra-day precision and accuracy for the determination of sitagliptin in six lots of human plasma.

Nominal conc. (ng/mL)	Mean measured conc.[a] (ng/mL, $n=6$)	Precision (RSD%, $n=6$)	Accuracy[b] (%)
1.00	0.99	6.1	99.0
2.00	2.05	2.9	102
5.00	5.04	3.2	101
20.0	20.1	3.1	100
100	103	1.4	103
500	493	1.8	98.6
800	790	1.7	98.8
1000	976	1.2	97.6

[a] Calculated from the weighted linear least-squares regression curve using all six replicates at each concentration.
[b] Expressed as (mean measured concentration/nominal concentration) × 100%.

Table 3
Precision and accuracy of QC samples for the determination of sitagliptin in human plasma.

Nominal conc. (ng/mL)	Mean measured conc.[a] (ng/mL, $n=5$)	Precision (RSD%)	Accuracy[b] (%)
3.00	2.91	1.2	97.0
100	104	0.67	104
800	793	0.81	99.1

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

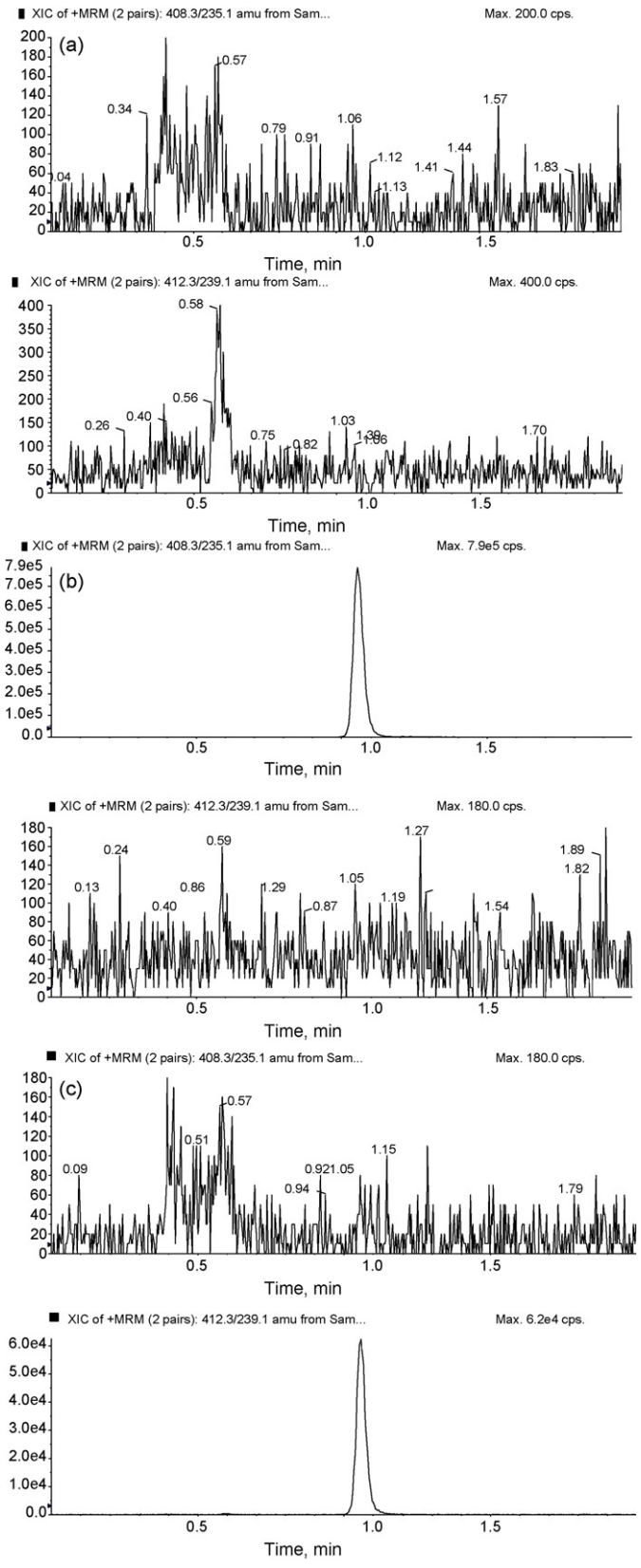

Fig. 3. Chromatograms of the plasma extract from (a) double blank, (b) 1000 ng/mL sitagliptin in the absence of IS, (c) single blank, and (d) 1.0 ng/mL standard sample (LLOQ). The top and bottom panels in each figure are the MRM of analyte and IS, respectively.

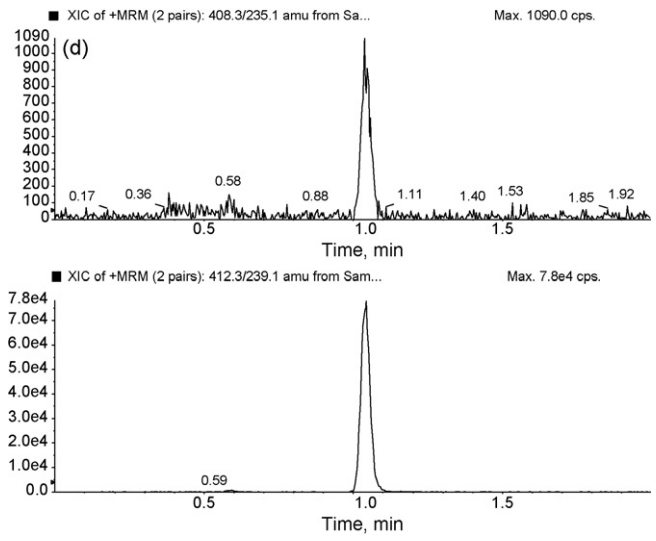

Fig. 3. (*Continued*).

3.5. Stability

Freeze–thaw stability for the plasma QC samples was evaluated using low, middle and high QCs that underwent three freeze–thaw cycles; QC samples at each concentration 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 QC samples that were subjected to three freeze–thaw cycles compared to the control QC concentrations (Table 5).

Room temperature stability was evaluated by analyzing QC samples exposed to ambient temperature for 17 h before being extracted. No significant calculated concentration differences were observed compared to the control QC concentrations (Table 5).

The long-term freezer storage stability of sitagliptin in human plasma was evaluated at −20 and −70 °C, respectively. The results indicated that sitagliptin in human plasma was stable for at least 13 months at −20 °C and 10 months at −70 °C. The longer storage time than 13 months at −20 °C and 10 months at −70 °C for sitagliptin in human plasma was not tested.

The stability of sitagliptin in whole blood has been investigated. The sitagliptin could be stable for at least 2 h in whole blood when it is placed on ice.

Table 4
Intra-day and inter-day precision and accuracy of LLOQ samples for the determination of sitagliptin in human plasma.

	Day 1	Day 2	Day 3
1	1.01	0.91	0.99
2	0.95	0.98	0.95
3	0.96	0.88	0.94
4	0.92	0.96	1.06
5	0.89	0.99	1.01
Intra-day mean ($n = 5$)	0.95	0.94	0.99
Intra-day precision (RSD%)	4.8	5.0	4.9
Intra-day accuracy[a] (%)	94.6	94.4	99.0
Inter-day mean ($n = 3$)		0.96	
Inter-day precision (RSD%)		2.8	
Inter-day accuracy[a] (%)		96.0	

[a] Expressed as [(mean measured concentration)/(nominal concentration)] × 100%.

Table 5
Freeze–thaw and room temperature (17 h) stability of sitagliptin QC samples in human plasma.

Nominal conc. (ng/mL)	Control QC mean measured conc.[a] ($n = 5$)	3 F/T cycles mean measured conc.[a] ($n = 3$)	Difference from control[b] (%)	Room temp. mean measured conc.[a] ($n = 3$)	Difference from control[b] (%)
3.00	3.01 (4.0)	3.05 (2.6)	1.3	3.01 (1.7)	0.0
100	103 (2.0)	102 (1.4)	−1.0	103 (0.62)	0.0
1000	798 (2.6)	784 (0.79)	−1.8	795 (0.73)	−0.40

Numbers in parentheses are coefficients of variation (RSD%).
[a] Calculated from the weighted linear least-squares regression curve.
[b] Expressed as [(mean measured conc. – control QC mean measured conc.)/control QC mean measured conc.] × 100%.

3.6. Cross-validation

Cross-validation is a comparison of measured results generated from different analytical methods for the same samples (clinical samples or spiked matrix standards) [7]. Through the cross-validation, the comparability of the results generated by different analytical methods can be assessed. For the determination of sitagliptin in plasma, we previously reported on the use of a high turbulence liquid chromatography (HTLC) online extraction procedure for the determination of sitagliptin in plasma [3]; this method was established several years ago and was used to support the marketing registration of sitagliptin. Due to the previous use of the HTLC method, we were interested in determining how results obtained with the new assay compared with those obtained with the prior procedure.

Samples collected from six healthy subjects during a sitagliptin clinical study were used to compare results generated by the online and offline methods. Individual samples were initially analyzed using the online extraction method and samples from the six subjects were then pooled by time point and analyzed using the offline method. Using the online method data, the mean plasma concentration at each time point was calculated. These results were compared with the results generated from offline analysis of the pooled samples (Table 6). The results demonstrated that the differences between the measurements from the two methods were less than 3% (Table 6), indicating that the two methods provided comparable results.

3.7. Application of the method and incurred sample reanalysis

The offline method was successfully applied to the analysis of samples from a sitagliptin clinical study.

During method development and validation, the statistical parameters used to evaluate the quality of a newly developed method are generated based on the analysis of spiked matrix standards. Because the samples from dosed subject samples are more complex than spiked matrix samples, it is necessary to demonstrate assay reproducibility by using dosed subject samples (incurred sample reanalysis) [21].

Incurred sample reanalysis (ISR) was performed using 10% of the patient plasma samples from the study randomly selected and re-assayed in a separate batch run. For this study, 8 non-zero samples were randomly selected from 83 patient plasma samples. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 7% (Table 7), indicating good reproducibility of the offline HILIC method.

Table 6
Sitagliptin concentrations in clinical plasma samples determined using HTLC online extraction method and HILIC offline method.

Sample (post-dose)	Mean sample conc. (online method) (ng/mL, $n = 6$)	Pooled sample[a] conc. (offline method) (ng/mL, $n = 1$)	Difference[b] (%)
0.5 h	119	118	−0.84
1 h	287	282	−1.74
2 h	304	308	1.32
3 h	349	349	0.00
4 h	331	337	1.81
5 h	293	294	0.34
8 h	179	184	2.79
12 h	105	108	2.86

[a] Pooled sample was prepared by transferring equal volume of plasma sample from each of the six subjects at each time point into a tube and mix well.
[b] Expressed as [(pooled sample conc. – mean sample conc.)/mean sample conc.] × 100%.

Table 7
Incurred sample reanalysis using the HILIC offline method.

Sample	Initial conc. (ng/mL)	Re-assay conc. (ng/mL)	Difference[a] (%)
1	565.2	585.7	3.63
2	673.4	670.0	−0.50
3	215.3	219.0	1.72
4	307.3	327.9	6.70
5	339.1	325.5	−4.01
6	138.3	145.9	5.50
7	216.8	219.0	1.01
8	297.3	295.5	−0.61

[a] Expressed as [(re-assay conc. – initial conc.)/initial conc.] × 100%.

3.8. Assessment of interference from concomitant medications for the determination of sitagliptin concentrations

The sitagliptin target patient population is often treated with multiple medications including other hyperglycemic agents such as metformin and pioglitazone, hypercholesterimia treatments such as atorvastatin and simvastatin, as well as commonly used “over the counter” products. To assess the interference from concomitant medications for the determination of sitagliptin concentrations, 21 drugs or metabolites of drugs were selected and tested as 3 groups. Group 1 included pioglitazone (5 μg/mL), M-II hydroxy pioglitazone (5 μg/mL), simvastatin (5 μg/mL), simvastatin lactone (5 μg/mL) and metformin (5 μg/mL). Group 2 included atorvastatin (5 μg/mL), atorvastatin lactone (5 μg/mL), ortho-hydroxy atorvastatin (5 μg/mL), ortho-hydroxy atorvastatin lactone (5 μg/mL), para-hydroxy atorvastatin (5 μg/mL) and para-hydroxy atorvastatin lactone (5 μg/mL). Group 3 included 10 commonly used over-the-counter drugs; viz., acetaminophen (30 μg/mL), caffeine (10 μg/mL), ibuprofen (80 μg/mL), pheniramine (1 μg/mL), dextromethorphan (DXM, 3 ng/mL), nicotine (30 ng/mL), pseudoephedrine (350 ng/mL), diphenhydramine (100 ng/mL), acetylsalicylic acid (Aspirin, 30 μg/mL) and phenylephrine (60 ng/mL). These medications were added, at the specified concentrations, to sitagliptin low QC samples (co-med containing LQC). Control samples were prepared by adding the carrier solvent to the low QC samples. The peak area ratios of the co-med containing LQC ($n = 5$) and control LQC samples ($n = 5$) were then compared using the described method. The results indicate that the 21 tested drugs or metabolites of drugs did not interfere with the determination of sitagliptin under the assay conditions (Table 8).

Table 8
Assessment on interference from concomitant medications on the determination sitagliptin concentrations using HILIC offline method.

Concomitant medication group	Control QC sample (no co-med)		Test QC sample (with co-med)		Difference (%)
	Mean peak area ratio	RSD (%, $n = 5$)	Mean peak area ratio	RSD (%, $n = 5$)	
1	0.0331	1.1	0.0329	4.2	−0.6
2	0.0338	1.7	0.0382	2.3	13
3	0.0337	3.7	0.0323	2.7	−4.2

[a]Expressed as {[(mean peak area ratio of test QC) − (mean peak area ratio of control QC)]/mean peak area ratio of control QC} × 100%.

4. Conclusions

A simple, high-throughput offline LC–MS/MS method for the determination of sitagliptin in human plasma was developed and validated. The LLOQ of this method was 1.0 ng/mL using a 100 μL aliquot of plasma and the linear dynamic range of the calibration curve was 1.0–1000 ng/mL. The potential for matrix effects caused by phospholipids was effectively eliminated by filtering the supernatant from the protein precipitated sample through Hybrid-SPE-PPT plates. Compared to the previously described HTLC online extraction method, the HILIC offline assay was found to provide comparable results without specialized instrumentation and therefore, the method is more amenable for transfer between laboratories.

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