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Liquid chromatography and tandem mass spectrometry method for the quantitative determination of saxagliptin and its major pharmacologically active 5-monohydroxy metabolite in human plasma: Method validation and overcoming specific and non-specific binding at low concentrations

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ABSTRACT

A liquid chromatography and tandem mass spectrometry (LC–MS/MS) method was developed and validated to simultaneously determine the concentrations of saxagliptin (Onglyza™, BMS-477118) and its major active metabolite, 5-hydroxy saxagliptin to support pharmacokinetic analyses in clinical studies. The dynamic range of the assay was 0.1–50 ng/mL for saxagliptin and 0.2–100 ng/mL for 5-hydroxy saxagliptin. Protein precipitation (PPT) with acetonitrile was used to extract the analytes from plasma matrix before injecting on an Atlantis® dC18 column (50 mm × 2.1 mm, 5 μm) for LC–MS/MS analysis. The sample pre-treatment process was carefully controlled to disrupt DPP4-specific binding and non-specific binding observed at lower concentrations. The recoveries for both analytes were >90%. The assay was selective, rugged and reproducible; storage stability of at least 401 days at −20 °C was demonstrated. Under these chromatographic conditions, the isomers of saxagliptin and 5-hydroxy saxagliptin were chromatographically separated from saxagliptin and 5-hydroxy saxagliptin. The assay has been used to support multiple clinical studies and regulatory approvals.

1. Introduction

Saxagliptin (1*S*,3*S*,5*S*)-2-[(2*S*)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo [3.1.0]hexane-3-carbonitrile, BMS-477118, Onglyza™, Fig. 1 and also in the fixed dose combination of saxagliptin and metformin as Kombiglyze™ is an orally active, selective, long-acting and reversible dipeptidyl-peptidase 4 (DPP4) inhibitor that is marketed in USA, Europe and many other countries for the treatment of type 2 diabetes mellitus [1–6]. DPP4 inhibitors enhance levels of active glucagon-like peptide 1 (GLP-1) and other incretins, and facilitate glucose-dependent insulin secretion. In addition, GLP-1 inhibits glucagon release, slows gastric emptying, reduces appetite, and regulates the growth and differentiation of the insulin producting β cells in pancreatic islets [1].

To support saxagliptin clinical studies, several bioanalytical methods [7,8] were developed and validated using high performance liquid chromatography with tandem mass spectrometry (LC–MS/MS). Structures for saxagliptin and its active, monohydroxylated metabolite, 5-hydroxy saxagliptin, (1*S*,3*S*,5*S*)-2-[(2*S*)-2-amino-2-(3,5-di-hydroxy-1-adamantyl) acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile, are shown in Fig. 1. The plasma elimination half-life values of saxagliptin and 5-hydroxy saxagliptin are approximately 2.5 and 3.5 h [1], respectively, which is in contrast to a plasma DPP4 inhibition half-life of up to 24 h. It was proposed that pharmacologically active concentrations of saxagliptin and 5-hydroxy saxagliptin below the limits of quantitation of the available assays at the time (1 and 2 ng/mL for saxagliptin and 5-hydroxy saxagliptin, respectively)

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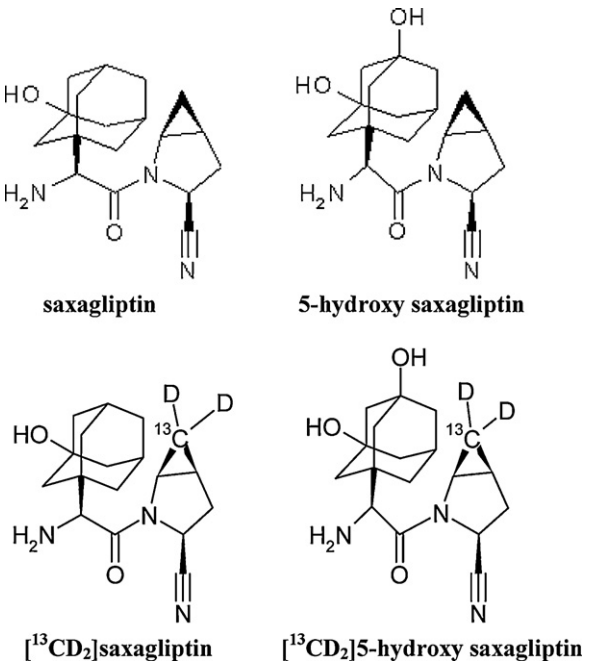

Fig. 1. Structure of saxagliptin (BMS-477118), 5-hydroxy saxagliptin and corresponding stable-isotope labeled internal standards.

were contributing to DPP4 inhibition in a secondary elimination phase that was not characterized with these earlier bioanalytical methods [7,8].

In this article, we describe efforts to develop and validate a simple, high-throughput LC–MS/MS method with a lower limit of quantification (LLOQ) in the sub-ng/mL range for the simultaneous quantification of saxagliptin and 5-hydroxy saxagliptin in human plasma, which could be utilized to better characterize a secondary pharmacokinetic elimination phase. The assay utilizes protein precipitation with CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) and sonication for sample preparation prior to LC–MS/MS analysis. The CHAPS treatment and sonication steps are critical to improve the recovery at lower concentration levels by disrupting DPP4-specific and non-specific absorption. Results of the assay validation, including assessment of precision and accuracy, QC stability, incurred sample reproducibility, potential interference from concomitant medicines, and cross-validation with an earlier assay are also discussed.

2. Experimental

2.1. Materials

Saxagliptin (Onglyza®, BMS-477118), 5-hydroxy saxagliptin, stable labeled saxagliptin ([$^{13}CD_2$]BMS-477118), stable labeled monohydoxylated metabolite ([$^{13}CD_2$]5-hydroxy saxagliptin) (Fig. 1) and the authentic standards for saxagliptin diastereomers (Fig. 2) were obtained from Bristol-Myers Squibb (Princeton, NJ, USA). After salt correction, the purities of saxagliptin and 5-hydroxy saxagliptin were 93.9% and 83.9%, respectively. HPLC-grade acetonitrile, methanol and ammonium hydroxide were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Formic acid (minimum purity 96%) was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA) and ammonium acetate was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, minimum purity 98%) was purchased from Sigma–Aldrich (St Louis, MO, USA). Water was obtained from an in-house Barnstead

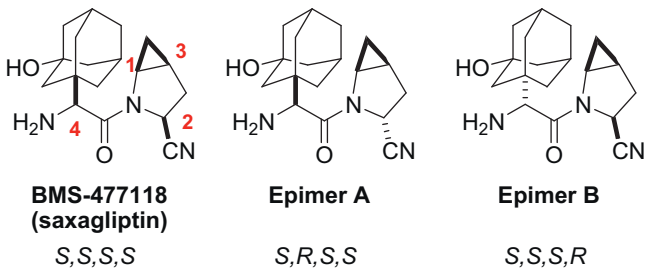

Fig. 2. Structure of saxagliptin and its stereoisomers.

De-ionized Water System (Billerica, MA, USA). Control human plasma (dipotassium EDTA) was purchased from Bioreclamation (NY, USA).

2.2. Instrumentation

The HPLC system consisted of a Shimadzu Autosampler (SIL-5000, set at ambient temperature), Shimadzu System Controller (Model SCL-10A Vp), Binary Pumps (Model LC 10AD Vp) and solvent degasser (Model DGU-14A) (Shimadzu Scientific Instrument, Columbia, MD, USA). A Sciex API 4000 mass spectrometer (Sciex, Toronto, Canada) was used as the detector. A Tomtec robotic liquid-handling system (Hamden, CT, USA) was used for the final transfer step in the sample preparation. Data were collected and processed using Analyst software v. 1.4 (Sciex, Toronto, Canada).

2.3. Chromatographic conditions

Saxagliptin and 5-hydroxy saxagliptin were separated on a 2.1 mm × 50 mm Atlantis® dC18 5 μm column (Waters, Milford, MA, USA). The chromatography was performed at room temperature, under gradient conditions. Mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Needle washes 1 and 2 were methanol:acetonitrile:formic acid (50:50:0.1, v/v/v) and water:acetonitrile:formic acid (70:30:0.1, v/v/v), respectively. Retention times of saxagliptin and 5-hydroxy saxagliptin were 2.3 and 1.4 min, respectively. A typical injection of 10 μL was sufficient to obtain required sensitivity. The typical cycle time is 6.0 min. The steps in Table 1 represent the typical mobile phase gradient program.

2.4. Mass spectrometer conditions

The mass spectrometer was a Sciex API-4000 equipped with a TurboIonSpray® source, operated in positive ionization mode, using selected reaction monitoring (SRM). The mass spectrometer was operated in unit resolution mode, with Q1 and Q3 set at 0.7 Da full width at half maximum (FWHM). Nitrogen was used as the nebulizer, curtain and collision gas. The optimized TurboIonSpray®

Table 1
HPLC gradient for mobile phase (A) and mobile phase (B).

Time from injection (min)	Total flowrate (mL/min)	% A	% B
0.01	0.300	96.0	4.00
3.00	0.300	70.0	30.0
3.50	0.300	5.00	95.0
3.51	0.300	5.00	95.0
3.52	0.600	5.00	95.0
4.50	0.600	5.00	95.0
4.51	0.300	5.00	95.0
4.52	0.300	5.00	95.0
4.53	0.300	96.0	4.00
6.00	0.300	96.0	4.00
6.01	Controller		Stop

and MS/MS conditions were as follows: 450 °C temperature setting for TIS interface, 65 setting for both GS1 and GS 2, 5000 V setting for ion spray voltage, 30 setting for the curtain gas and nebulizer gas. The declustering potentials (DP) were set to 50 V and 60 V for saxagliptin and 5-hydroxy saxagliptin, and the entrance potentials (EP) were set to 10 V for all analytes. The collision cell exit potential (CXP) was set at 15 for all analytes. The collision energy (CE) was set at 31 eV for all analytes.

Saxagliptin and 5-hydroxy saxagliptin gave an $[M+H]^+$ precursor ion of *m*/*z* 316 and 332, respectively. When each protonated precursor ion was selected for collision-activated dissociation (CAD) experiments, the predominant product ions are *m*/*z* 180 and 196, respectively. The internal standards, $[^{13}CD_2]$saxagliptin and $[^{13}CD_2]$5-hydroxy saxagliptin, gave an $[M+H]^+$ precursor ion of *m*/*z* of 319.2 and 335.3, respectively. The fragment ions from the CAD experiments were *m*/*z* 180.3 and 196.3, respectively. The precursor-to-product ion transitions used were 316.2 → 180.2 and 332.3 → 196.3 for saxagliptin and 5-hydroxy saxagliptin, respectively. The described precursor and fragment ions transitions were used for the internal standards. The dwell time for each transition was 50 ms.

2.5. Standards and quality controls (QCs)

Two sets of stock solutions (1.00 mg/mL prepared with acetonitrile:water (50:50, v/v)) of saxagliptin and 5-hydroxy saxagliptin were prepared from independent weighings. Once agreement between the solutions had been verified, one stock solution was used for calibration standards and the other was used for quality control samples. A correction for the impurity of the standards was made when preparing stock solutions.

Intermediate stock solutions with concentrations of 10.0 and 20.0 μg/mL of saxagliptin and 5-hydroxy saxagliptin, respectively, were prepared with acetonitrile:water (50:50, v/v) from the 1.00 mg/mL stock solution. Primary stock solutions and all intermediate stock solutions were stored at 1–8 °C for up to 5 months (data not shown). Calibration standard were prepared freshly in blank K_2EDTA plasma on the day of use. Nominal saxagliptin/5-hydroxy saxagliptin concentrations were 0.10/0.20, 0.20/0.40, 0.40/0.80, 1.0/2.0, 4.0/8.0, 10.0/20.0, 20.0/40.0, 45.0/90.0, and 50.0/100 ng/mL, respectively. Duplicate sets of standards were used to bracket each analytical run, with one curve before and after the QCs and study samples.

QC samples were prepared with similar procedures to those described for preparation of the standard. Nominal QC concentrations were 0.30/0.60, 0.60/1.20, 3.00/6.00, 6.0/12.0, 40.0/80.0, and 500/1000 ng/mL in plasma, respectively. QC pools were subdivided following preparation in single use portions and frozen at −20 °C.

Internal standard working solutions for $[^{13}CD_2]$saxagliptin and $[^{13}CD_2]$5-hydroxy saxagliptin were prepared from 1.00 mg/mL stock solutions in acetonitrile:water (50:50, v/v). From the 1.00 mg/mL stock solution, the combined $[^{13}CD_2]$saxagliptin/$[^{13}CD_2]$5-hydroxy saxagliptin intermediate internal standard solution is prepared at 5000/10,000 ng/mL in acetonitrile:water (50:50, v/v). From the above intermediate stock solution, the combined internal standard working solution is prepared at 5.0/10.0 ng/mL in acetonitrile:water (50:50, v/v). Stock, intermediate and working solution stabilities have been demonstrated for up to 90 days at 1–8 °C.

2.6. Sample preparation

Human plasma samples were thawed at room temperature and vortex-mixed to ensure uniformity prior to transfer. A 100 μL volume of each sample was transferred to a 96-well polypropylene plate. A 10 μL portion of 10% CHAPS (prepared in water) was added into each of the above samples, this was immediately followed by a 45 min sonication at room temperature. Samples were then heated for approximately 5 min at 55 °C on a plate heater. Following pretreatment, a 50 μL volume of working internal standard solution was added to each sample except double blanks. Samples were precipitated with 500 μL of acetonitrile; vortexed for 2 min; and centrifuged at 3000 rpm for 5 min at room temperature. The Tomtec was used to transfer 400 μL of the resulting supernatant to a new 96-well polypropylene plate. The mixture was evaporated to dryness at 50 °C. Following addition of a 200 μL of reconstitution solution consisting of acetonitrile:water: formic acid (4:95:0.1, v/v/v), the 96-well plate was vortexed mixed and placed in the autosampler; and 10 μL was injected onto the column for analysis.

2.7. Method validation

A standard curve was constructed from the peak area ratios of saxaglipin and 5-hydroxy saxagliptin to their respective internal standards *vs.* the nominal concentration of the standards. Unknown plasma sample concentrations were calculated from the equation ($y = ax + b$) as determined by the weighted ($1/x^2$) linear regression model; where x is the nominal concentration of saxaglipin or 5-hydroxy saxagliptin.

Validation of the method with respect to accuracy and precision was carried out according to the FDA Bioanalytical Method Validation Guidance for Industry [9] and the Crystal City III white paper [10]. The accuracy and precision of the method was assessed by analyzing six QC samples (prepared in blank plasma) in every run among the 4 accuracy and precision runs at saxagliptin and 5-hydroxy saxagliptin concentrations of 0.10/0.20 (LLOQ), 0.30/0.60, 0.60/1.20, 3.00/6.00, 6.0/12.0, 40.0/80.0 and 500/1000 ng/mL. The saxagliptin/5-hydroxy saxagliptin plasma QC sample of 500/1000 ng/mL was used to establish dilution integrity and was diluted 20-fold with blank matrix prior to processing. Six replicate samples at each concentration were analyzed in three separate runs. The accuracy was determined by calculating the percentage deviations (%Dev) of the predicted concentrations from their nominal values. The intra- and inter-run assay precision was determined by calculating the %CV values.

To evaluate bench top stability, human K_2EDTA plasma saxagliptin/5-hydroxy saxagliptin quality control (QC) samples at 0.30/0.60 and 15.0/30.0 ng/mL were kept at room temperature for 24 h prior to extraction. Additionally, saxagliptin/5-hydroxy saxagliptin QC samples at 0.30/0.60, 40.0/80.0 and 500/1000 ng/mL were also kept at room temperature for 42 h prior to extraction. The saxagliptin/5-hydroxy saxagliptin quality control sample at 500/1000 ng/mL was diluted 20-fold with blank matrix just prior to extraction. At the indicated time points, the samples were extracted and analyzed by comparison to freshly prepared standard curves. The deviations of the mean predicted concentrations of the test QC samples from the nominal concentrations were used as an indicator of the room temperature stability of saxagliptin and 5-hydroxy saxagliptin in human K_2EDTA plasma.

The freeze–thaw stability of saxagliptin and 5-hydroxy saxagliptin in human K_2EDTA plasma was assessed over six freeze (−20 °C)/thaw (room temperature) cycles using QC samples at 0.30/0.60, 40.0/80.0 and 500/1000 ng/mL, in a manner consistent with typical sample analysis. After the completion of six cycles, the plasma QC sample at 500/1000 ng/mL was diluted 20-fold with blank human plasma and extracted along with the other freeze/thaw QC samples.

The saxagliptin/5-hydroxy saxagliptin extract stability of the processed samples (autosampler stability) was assessed for 31 h at room temperature using QC samples at 0.30/0.60, 3.00/6.00, 20.0/40.0 and 40.0/80.0. These QCs were extracted and analyzed along with a freshly prepared duplicate standard curve at time 0.

The extracted QC samples remained in the autosampler for 31 h until they were reinjected along with a second freshly extracted duplicate calibration curve and were compared against nominal values to obtain the measured saxagliptin/5-hydroxy saxagliptin concentrations.

Reinjection integrity was assessed for 146 h at room temperature and normal lighting conditions after original sample analysis using saxagliptin/5-hydroxy saxagliptin QC samples at 0.30/0.60, 40.0/80.0 and 500/1000 ng/mL. Following extraction, the processed QCs were analyzed against a duplicate standard curve. After the successful completion of the initial analysis, the processed standard curve samples and QC samples were stored at room temperature for 146 h from their first injection prior to reinjection. The reinjected QC samples were quantitated against the reinjected standard curves and compared to nominal values.

The extraction efficiency and matrix effect for saxagliptin/5-hydroxy saxagliptin in human plasma, expressed as a percentage, was determined at 0.30/0.60 and 40.0/80.0 ng/mL by comparing three sets of samples: ((A) drug spiked to plasma and prepared normally (pre-extraction) (B) drug spiked after extraction of blank plasma (post-extraction); and (C) drug spiked directly into reconstitution solvent (4:96:0.1, v/v/v, acetonitrile:water:formic acid) with no prior extraction. The matrix effect was calculated as the percentage decline in response between B and C. Extraction efficiency was calculated as the response ratio of A/B. The recovery of the IS was determined similarly (mean peak area ratio (internal standard/analyte)) at the level of use.

2.8. Incurred QC (IQC) preparation

To ensure the quality of the chromatographic separation was maintained throughout the validation and sample analysis runs for 5-hydroxy saxagliptin and minor isomers present in low concentrations, system suitability samples made from incurred samples (designated as incurred QC samples, IQC) were placed at the beginning and the end of each run. IQCs were prepared from extracts of microsomal incubations (rat, monkey, or human) and were known to contain saxagliptin, 5-hydroxy saxagliptin, and additional monohydroxylated metabolites (such as M1, M3 and M13) [11]. The IQC solution was prepared in bulk by diluting 20 μL of microsomal extract with 20 mL of water:acetonitrile:formic acid (96:4:0.1, v/v/v). The extracts were stored at 1–8 °C. Chromatograms from a micrsomal extract injection are shown in Fig. 3.

2.9. Assay applications

The assay was applied to multiple bioequivalence studies. Here, we present results from a bioequivalence study for a 2.5 saxagliptin/1000 mg metformin immediate release fixed-dose combination (FDC) formulation (Kombiglyze™) where the pharmacokinetics of saxagliptin and 5-hydroxy saxagliptin following dosing of the FDC were compared to the individual components (2.5 mg Onglyza™ and 1000 mg Glucophage™) in healthy subjects ($n = 19$).

3. Results and discussions

3.1. Challenges in assay development due to the major active metabolite and its regioisomers

The metabolism of saxagliptin is primarily mediated by cytochrome P450 3A4/5 (CYP3A4/5)[11]. Multiple hydroxylated metabolites were formed through this pathway, including 5-hydroxy saxagliptin, which is the major circulating metabolite. 5-Hydroxy saxagliptin is pharmacologically active, with approximately one-half of the *in vitro* DPP4 inhibitory potency of the parent drug [1]. In addition to 5-hydroxy saxagliptin, there are at least three monohydroxylated regioisomer metabolites. These metabolites have the same SRM transitions, and, therefore, require chromatographic resolution to minimize interference. The HPLC separation conditions reported here are capable of successfully separating the other monohydroxylated metabolites from 5-hydroxy saxagliptin (Fig. 3). Although there is no quantitative acceptance criteria for separation, the separation is considered acceptable when baseline resolution of the peaks were obtained and maintained for all peaks in the m/z 332.3 → 196.3 channel as shown in Fig. 3.

3.2. The strategy for separating potential saxagliptin stereoisomers

Saxagliptin has four epimerizable stereogenic centers (*S*,*S*,*S*,*S* configuration). Eight (8) possible diastereomers, including saxagliptin itself, are theoretically possible. The formation of any of these diastereomers is not anticipated to occur with saxagliptin through metabolic chiral inversion (*i.e.*, oxidation of a secondary alcohol, conjugation of a carboxylic acid with acetyl CoA) [12]. However, it is possible that chiral inversion may occur through chemical mechanisms, either *in vivo* or *ex vivo* during sample storage or processing. The formation of epimer A (*S*,*R*,*S*,*S*) and epimer B (*S*,*S*,*S*,*R*) (Fig. 2) are more likely than any of the others because they are each the product of inversion at only one of the two independent stereogenic sites. Formation of the other diastereomers is considered unlikely as they would require either inversion of both centers defined by the cyclopropyl ring or sequential inversion at multiple chiral sites of the saxagliptin molecule.

To investigate whether the diastereomers were present in humans, the authentic standards were injected onto the LC–MS system used for metabolic profiling of the samples from the human ADME study [13] with [^{14}C]saxagliptin. Under these conditions, each of the available diastereomers was baseline resolved from the saxagliptin peak. The chromatograms from human plasma and urine samples were then re-examined for the presence of radioactivity or MS signal at the retention times corresponding to the authentic standards. There was no evidence for any signal by either method, thus it was concluded that these components were not present in these human plasma samples. Detailed data will be discussed in a separate manuscript. To further investigate the possibility for formation of diastereomers of saxagliptin, specifically, epimer B which is most likely to form under plasma sample preparation conditions, a mixture of saxagliptin and epimer B was analyzed under the same condition used in the validated assay for saxagliptin. In this system, epimer B was found to be baseline resolved from saxagliptin. As above, the mass spectral chromatograms for several clinical study samples were examined for the presence of a peak corresponding to epimer B, but no signal was detected. Additionally, QC plasma samples through bench-top and frozen storage were also examined for the potential presence of diastereomers, no signal was detected either. Overall, experimental data described above demonstrated, if present at all, the products of chiral inversion of saxagliptin are present in very small quantities. The combined evidence led to the conclusion that saxagliptin does not undergo any significant chiral inversion after administration to human.

3.3. Cross-validation for current assay with lower LLOQ and the previous assay

Two different validated assays were used to support advanced clinical studies. The original assay had a lower limit of quantification of (LLOQ) 1.0/2.0 ng/mL for saxagliptin/5-hydroxy saxagliptin,

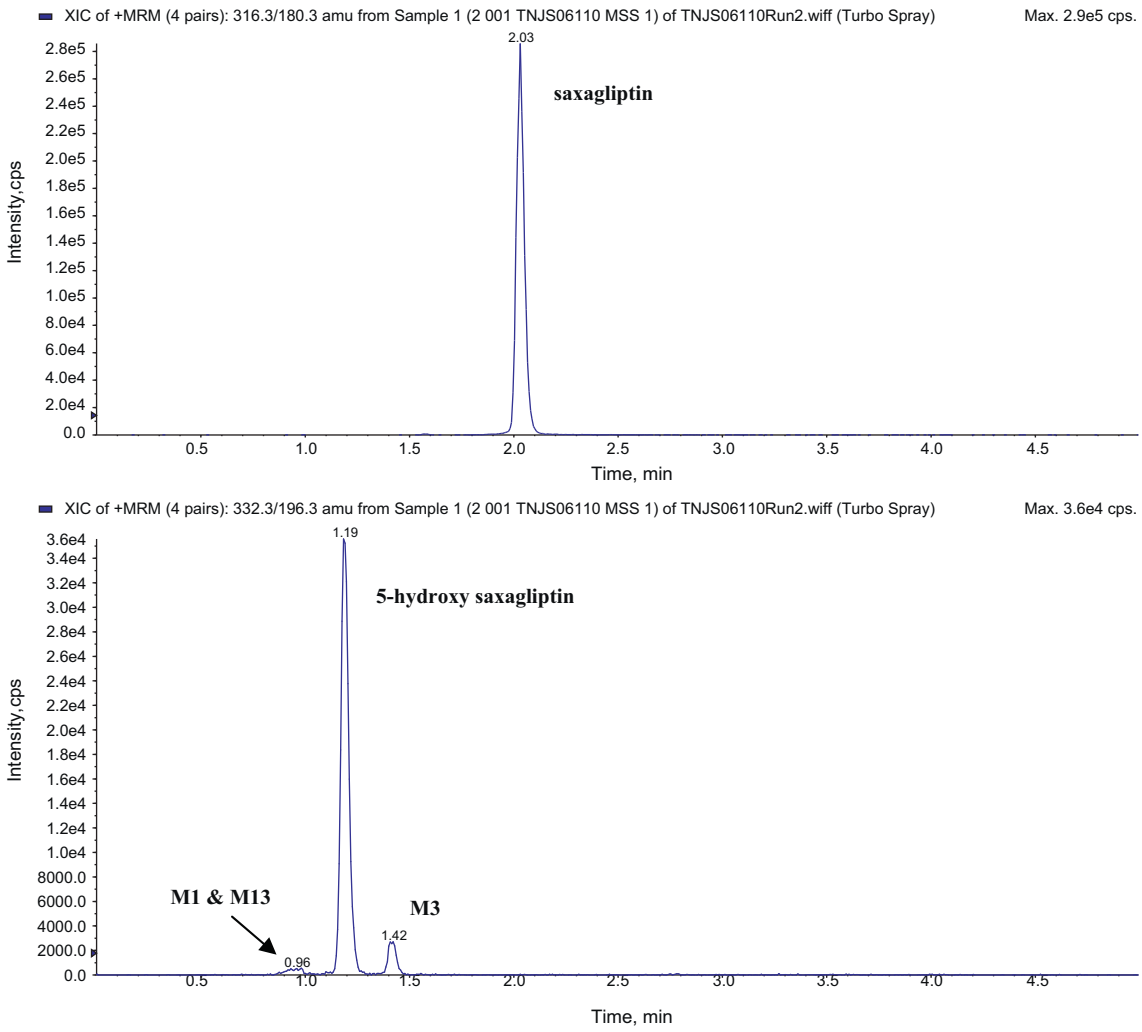

Fig. 3. Representative chromatograms of microsomal extract, showing resolution of other hydroxy metabolites from 5-hydroxy saxagliptin.

respectively. A second assay, the current assay described in this manuscript, which lowered the limit of quantification of (LLOQ) to 0.10 ng/mL/0.20 ng/mL for saxagliptin/5-hydroxy saxagliptin, respectively, was developed to fully characterize the terminal phase of pharmacokinetic elimination. The two validated assays were cross-validated by analyzing pooled incurred samples and QCs by each method and comparing the results. The two validated methods were found to be equivalent. Summaries of the assay validation parameters including accuracy and precision are provided in Table 2. The cross-validation fulfilled all requirements and recommendations regarding linearity, precision, accuracy, sensitivity and selectivity.

3.4. Challenges for method development of a lower LLOQ assay

During the method development and mock validation for the low curve range assay, LLOQ QC and low QC samples were not reproducible from run to run. Experimental data (data not shown) demonstrated that a negative bias from nominal values at 50% for LLOQ QCs and 30–50% for Low QCs was common. At room temperature, within 1 h of preparation, LLOQ QC and low QC loss against nominal values were between 20 and 30%. The negative bias became more prominent after LLOQ and low QCs were stored at −20 °C (*vs.* freshly prepared). The phenomena were not observed in the assay with the higher analytical range. At low concentration levels of 0.1/0.3 ng/mL and 0.2/0.6 ng/mL for saxagliptin and 5-hydroxy saxagliptin, respectively, nonspecific binding to containers and DPP4-specific binding/dissociation were suggested to be the cause for the loss of recovery. Adding a surfactant (10 μL of 10% CHAPS) modestly improved the poor recovery by 20–30% for both analytes, which is about 70–80% of what was lost. Sonicating samples at room temperature (RT) for 45 min immediately after adding CHAPS and maintaining the sample plate on a warming plate for 2–5 min further improved the recovery an additional 20–30%, which eliminated all loss and returned measured concentrations back to nominal levels. Based on these observations, the final procedure incorporates the addition of CHAPS followed by sonicating samples at room temperature for 45 min and heating the sample plate or 2–5 min prior to extraction.

The plasma concentration of DPP4 is estimated to be 1–5 nM [14]. Below this concentration, majority of the saxagliptin and 5-hydroxy saxagliptin are bound to DDP4; it is estimated as much as 30% of the saxagliptin and 50% of 5-hydroxy saxagliptin may be bound at this concentration. Sonicating and heating the sample plate rapidly disrupts the binding of saxagliptin and 5-hydroxysaxagliptin to the DPP4 and therefore facilitate the dissociation of analytes from DPP4 binding.

3.5. Assay validation summary

The assay with lower LLOQ was fully validated to ensure the quality of clinical data. Assay selectivity was established by the

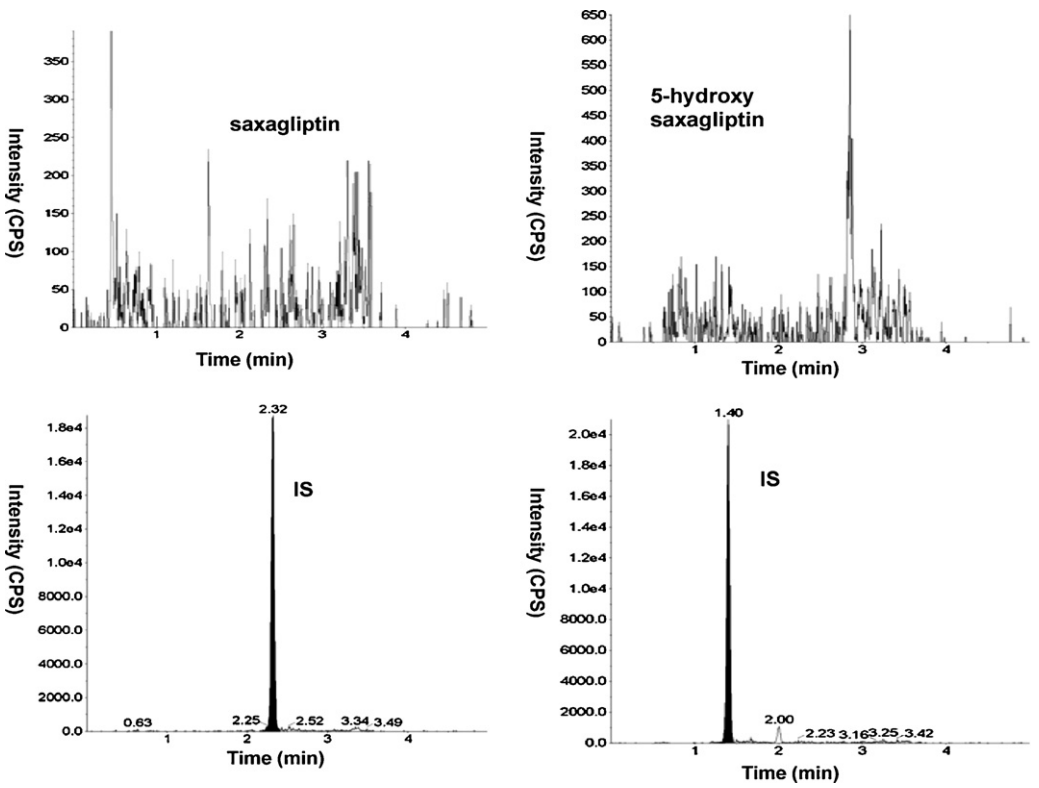

Fig. 4. Representative chromatogram of blank human plasma with internal standard (IS) for saxagliptin and 5-hydroxy saxagliptin.

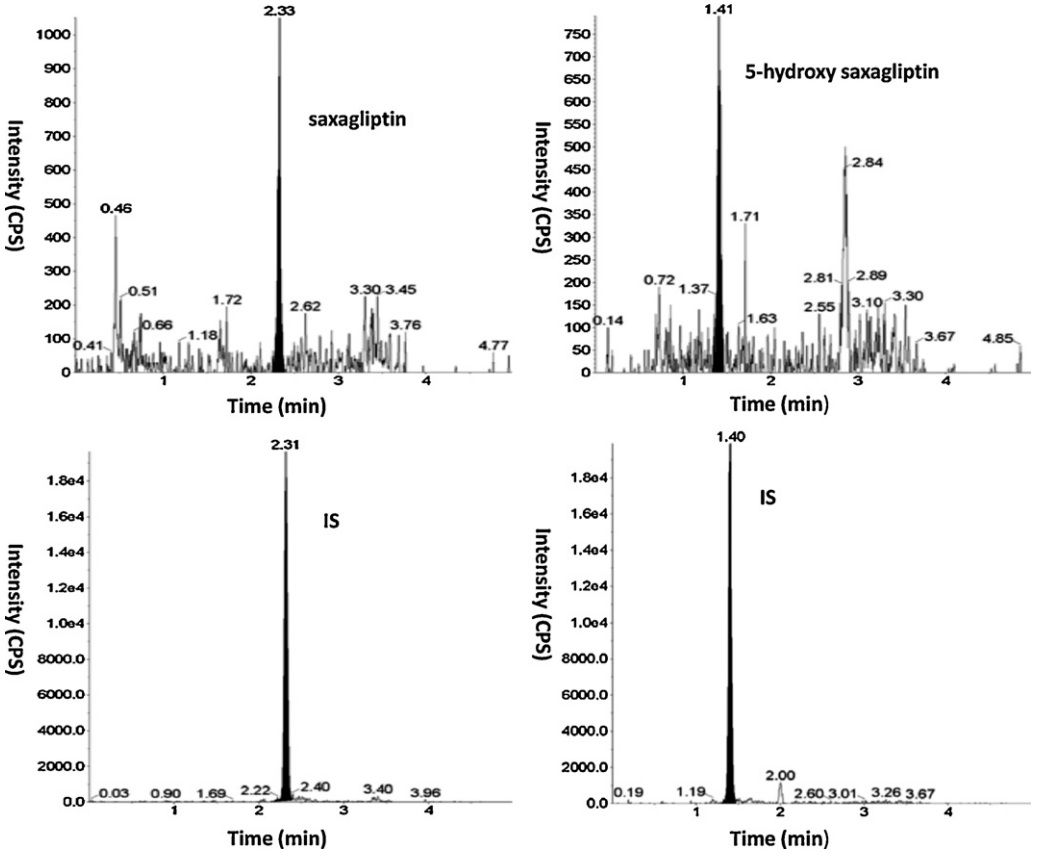

Fig. 5. Representative chromatograms of a plasma sample spiked with saxagliptin and 5-hydroxy saxagliptin at the lower limit of quantitation (LLOQ) (nominal = 0.10 ng/mL and 0.20 ng/mL).

Table 2
Cross-validation results for saxagliptin and 5-hydroxy saxagliptin in human plasma with an earlier validated assay. All concentrations are expressed as ng/mL.

Sample	Method	Run	Saxagliptin			5-Hydroxysaaxgliptin		
			Mean of replicates	%CV of replicates	%Deviation[a] between early method (Method 1) and current method (Method 2)	Mean of replicates	%CV of replicates	%Deviation[a] between Method 1 and Method 2
QC A	Method 2	7	20.7	7.8		42.3	9.3	
	Method 1	15	20.3	1.9	1.1	40.5	5.2	2.2
QC B	Method 2	7	0.310	11.5		0.635	9.6	
	Method 1	15	N/A	N/A	N/A	N/A	N/A	N/A
QC C	Method 2	7	3.17	1.6		6.56	5.7	
	Method 1	15	3.18	3.8	0.3	6.43	10.7	1.0
QC D	Method 2	7	40.0	1.1		80.3	1.3	
	Method 1	15	40.4	1.7	0.5	81.8	2.7	0.9
Sample A	Method 2	7	15.4	1.1		18.6	3.0	
	Method 1	14	14.5	11.2	2.9	18.1	15.0	1.5
	Method 1	15	15.5	3.2	0.2	19.0	3.1	0.9
Sample C	Method 2	7	13.8	1.5		44.5	0.6	
	Method 1	14	12.6	5.2	4.3	42.0	7.9	2.9
	Method 1	15	14.0	2.1	0.8	44.4	3.0	0.0
Sample D	Method 2	7	12.1	6.7		40.1	6.0	
	Method 1	14	11.5	3.0	2.4	38.4	5.5	2.1
	Method 1	15	11.7	2.8	1.7	40.9	6.4	1.0
Sample F	Method 2	7	17.3	0.9		65.0	2.9	
	Method 1	14	16.2	10.2	3.4	64.9	12.1	0.1
	Method 1	15	17.4	3.8	0.1	69.3	3.6	3.2

[a] %Deviation = absolute value of: (mean of Method 1 replicates − ((mean of Method 1 replicates + mean of Method 2 replicates)/2))/(mean of Method 1 replicates + mean of Method 2 replicates).

separation of the diastereomers from saxagliptin and all other hydroxylated saxagliptin metabolites from 5-hydroxy saxagliptin. The assay selectivity was established by analyzing six different lots of K_2EDTA human control plasma spiked with and without internal standard and measuring the peak response at expected retention time of saxagliptin and 5-hydrox saxagliptin. A representative example of these assessments is shown in Fig. 4. No significant interferences were found at the retention times of either saxagliptin or 5-hydroxy saxagliptin. A representative chromatogram at LLOQ level is shown in Fig. 5. Table 3 presents the summary of the standard curve data obtained in the four runs on four days used to validate the method. The precision and accuracy of the standard curves, at each concentration, were within ±15.0% including the lower limit of quantitation (LLOQ), which has an acceptance limit of ±20.0%.

Table 4 shows the precision and accuracy of QC samples for the determination of saxagliptin and 5-hydroxy saxagliptin in human plasma. The assay precision for saxagliptin and 5-hydroxy saxagliptin were within 8.2% CV and 8.2% CV, respectively.

The bench-top stability of saxagliptin and 5-hydroxy saxagliptin in human K_2EDTA plasma was evaluated for up to 42 h at room temperature. Saxagliptin/5-hydroxy saxagliptin stability QC samples were kept at room temperature for either 24 h or 42 h prior to extraction. At the indicated time point, the samples were extracted and analyzed by comparison to freshly-prepared standard curves. As shown in Table 5, saxagliptin and 5-hydroxy saxagliptin in human plasma were determined to be stable for at least 42 h at room temperature with the exception that saxagliptin at low QC level (0.3 ng/mL) was only stable for 24 h. The change in dissociation equilibrium from DDP4 for saxagliptin at lower temperature could be one reason for the apparent instability at the QC low level.

The freeze–thaw stabilities of saxagliptin and 5-hydroxy saxagliptin in human K_2EDTA plasma were demonstrated over six freeze (−20 °C)/thaw (room temperature) cycles, as shown in Table 5, using QC plasma samples.

The long-term storage stability of saxagliptin and 5-hydroxy saxagliptin in human plasma was demonstrated for up to 401 days at −20 °C, as shown in Table 5, using QC plasma samples. The stability QC samples were analyzed against a freshly prepared standard curve.

The processed sample stability (autosampler stability) was demonstrated for 31 h at room temperature after original sample

Table 3
Summary of standards for saxagliptin and 5-hydroxy saxagliptin from accuracy and precision runs.

Saxagliptin	0.100 ng/mL	0.200 ng/mL	0.400 ng/mL	1.00 ng/mL	4.00 ng/mL	10.0 ng/mL	20.0 ng/mL	45.0 ng/mL	50.0 ng/mL
Mean	0.098	0.205	0.418	1.00	4.07	10.4	19.8	43.0	47.5
SD	0.012	0.010	0.030	0.074	0.103	0.361	0.383	2.204	1.499
CV%	12.5	4.9	7.1	7.4	2.5	3.4	1.9	5.1	3.2
Deviation	−2.27	2.25	4.50	0.10	1.71	3.58	−1.25	−4.33	−4.92
N	6	6	6	6	6	6	6	6	5

5-Hydroxy saxagliptin	0.200 ng/mL	0.400 ng/mL	0.800 ng/mL	2.00 ng/mL	8.00 ng/mL	20.0 ng/mL	40.0 ng/mL	90.0 ng/mL	100 ng/mL
Mean	0.200	0.393	0.816	2.04	8.00	20.6	39.4	88.8	97.3
SD	0.011	0.038	0.050	0.094	0.357	0.668	1.419	1.978	1.692
CV%	5.4	9.8	6.1	4.6	4.5	3.3	3.6	2.2	1.7
Deviation	0.08	−1.70	2.00	2.25	−0.06	2.33	−1.54	−1.31	−2.72
N	6	5	6	6	6	6	6	6	5

Table 4
Accuracy and precision for saxagliptin and 5-hydroxy saxagliptin quality control (QC) samples in human K_2EDTA plasma.

Saxagliptin	LLOQ QC 0.1 ng/mL	Low QC 0.3 ng/mL	GM[b] QC 3 ng/mL	Mid QC 20 ng/mL	High QC 40 ng/mL	Dilution QC 500 ng/mL (20×[c])
Mean	0.105	0.299	3.02	19.9	38.7	492
SD	0.0086	0.0228	0.1178	0.6002	1.598	21.08
%CV[a]	8.2	7.6	3.9	3.0	4.1	4.3
%Nominal	104.5	99.6	100.6	99.4	96.7	98.4
%Deviation	4.5	−0.4	0.6	−0.6	−3.3	−1.6

5-Hydroxy saxagliptin	LLOQ QC 0.2 ng/mL	Low QC 0.6 ng/mL	GM[b] QC 6 ng/mL	Medium QC 40 ng/mL	High QC 80 ng/mL	Dilution QC 1000 ng/mL (20×[c])
Mean	0.187	0.578	6.06	39.8	78.6	985
SD	0.0154	0.0285	0.3431	1.9099	3.376	50.09
%CV[a]	8.2	4.9	5.7	4.8	4.3	5.1
%Nominal	93.5	96.4	100.9	99.6	98.2	98.5
%Deviation	−6.5	−3.6	0.9	−0.4	−1.8	−1.5

[a] Data summary are based on inter-run data from 4 accuracy/precision runs.
[b] Geometrical mean.
[c] Diluted 20-fold with control human plasma prior to analysis.

analysis using QC samples. The reinjected QC samples were quantitated against the original standard curve and new regression. The deviations of the predicted concentrations of the reinjected QC samples from their nominal values ranged from 2.0% to 6.0% for saxagliptin, and from 1.2% to 4.5% for 5-hydroxy saxagliptin.

The reinjection integrity was demonstrated for 146 h at room temperature after original sample analysis using QC samples. The reinjected QC samples were quantitated against standard curves that were processed and reinjected along with these QC samples. The deviations of the predicted concentrations of the reinjected QC samples from their nominal values ranged from −3.7% to 0.3% for saxagliptin, and from −1.0% to 6.0% for 5-hydroxy saxagliptin.

The extraction efficiency and matrix effect for saxagliptin in human plasma, expressed as a percentage, was determined at 0.30 and 40.0 ng/mL. The recovery of saxagliptin was 98.5 and 99.0% at the levels tested and that of the IS was 102.5 and 106.6%. The results from the matrix effects evaluation for saxagliptin were 1.15 and 1.20 at the levels tested and that of the IS was 1.15 and 1.17.

The extraction efficiency and matrix effect for 5-hydroxy saxagliptin in human plasma, expressed as a percentage, was determined at 0.60 and 80.0 ng/mL. The recovery of 5-hydroxy saxagliptin was 97.1 and 97.8% at the levels tested and that of its IS was 110.2 and 104.0%. The results from the matrix effects evaluation for 5-hydroxy saxagliptin were 1.09 and 1.17 at the levels tested and that of the IS was 1.07 and 1.19.

3.6. Application to the PK of saxagliptin and 5-hydroxy saxagliptin following oral (PO) administration of saxagliptin

The validated method with lower LLOQs was applied to the pharmacokinetics of saxagliptin and 5-hydroxy saxagliptin in late phase and life-cycle management clinical studies employing a low dose (2.5 mg) PO fixed dose combination (FDC) formulation with metformin (1000 mg). Appropriate written informed consent was obtained from the subjects participating in the clinical trial and that ethical guidelines were followed. The effect of combinational metformin on the quantification of saxagliptin and 5-OH saxagliptin was assessed by demonstrating the long term stability of saxagliptin and 5-OH saxagliptin plasma QCs for up to 6 months at −20 °C with the presence of metformin in the long term stability

Table 5
Stability data from saxagliptin and 5-hydroxy saxagliptin QC samples in human K_2EDTA plasma.

	Nominal conc. (ng/mL)	Mean found conc. (ng/mL)[a]	Precision (%CV)	Accuracy (%Dev)[b]
Saxagliptin				
Bench-top stability, 24 h, $n = 6$	0.300	0.269	12.5	−10.3
Bench-top stability, 42 h, $n = 6$	40.0	38.6	2.4	−3.5
	500 (DF[c] = 20)	481	1.1	−2.6
Freeze/thaw stability at −20 °C, 6 cycles, $n = 6$	0.300	0.286	6.7	−4.7
	40.0	40.3	0.9	0.8
	500 (DF = 20)	485	1.9	−3.0
Long term stability at −20 °C, 401 days, $n = 3$	0.300	0.295	6.8	−1.7
	40.0	37.8	2.2	−5.5
	500 (DF = 10)	495	3.5	−1.0
5-Hydroxy saxagliptin				
Bench-top stability, 42 h, $n = 6$	0.600	0.570	8.3	−5.0
	80.0	77.8	6.0	−2.8
	1000 (DF = 20)	979	4.7	−2.1
Freeze/thaw stability at −20 °C, 6 cycles, $n = 6$	0.600	0.599	5.6	−0.2
	80.0	79.5	1.6	−0.6
	1000 (DF = 20)	969	1.3	−3.1
Long term stability at −20 °C, 401 days, $n = 3$	0.600	0.580	6.5	−3.3
	80.0	76.7	1.4	−4.1
	1000 (DF = 10)	993	3.1	−0.7

[a] Calculated from the weighted least-square regression curve, mean values are reported here.
[b] Expressed as [(mean found concentration − nominal concentration)/nominal concentration)] × 100%.
[c] DF: dilution factor, QCs were diluted with appropriate fold with control human plasma prior to analysis.

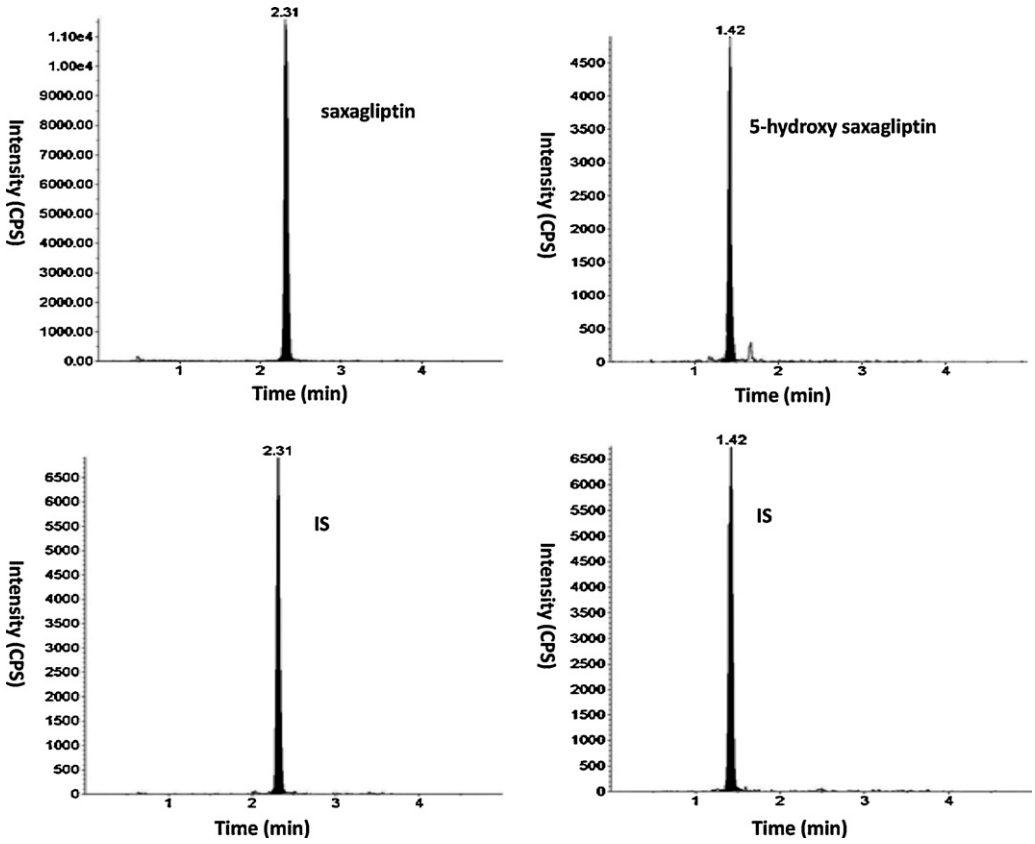

Fig. 6. Typical chromatograms for saxagliptin and 5-hydroxy saxagliptin in a plasma sample from a patient dosed orally with saxagliptin 2.5 mg. Calculated concentration of 3.14 ng/mL for saxagliptin and 2.65 ng/mL for 5-hydroxy saxagliptin.

QCs. Therefore, no interference from metformin on the quatification of saxagliptin and 5-hydroxy saxagliptin was observed in the assay at typical clinical plasma exposures. A typical chromatogram of a healthy subject sample is presented in Fig. 6 with calculated concentrations of 3.14 ng/mL and 2.65 ng/mL for saxagliptin and 5-hydroxy saxagliptin, respectively. Fig. 7 shows the mean (+SD) saxagliptin and 5-hydroxy saxagliptin plasma concentration time profile following the 2.5 mg of saxagliptin oral dose. At this dose, the pharmacokinetics of saxagliptin could be followed for 24 h with the new, more sensitive assay (0.1 ng/mL LLOQ) as opposed to ≤12 h with the previous assay (1 ng/mL). Furthermore, a second elimination phase is evident in the plasma pharmacokinetic profiles of both saxagliptin and 5-hydroxy saxagliptin (mean elimination phase half-live values of 6.7 and 8.1 h, respectively) that had not been previously characterized.

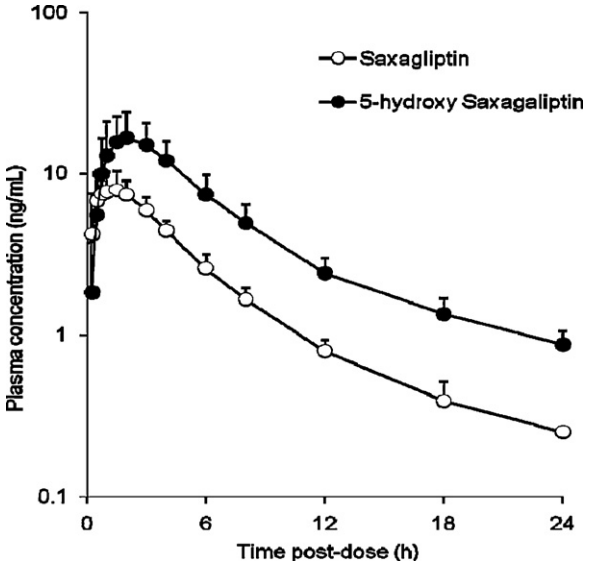

Fig. 7. Mean (+SD) saxagliptin and 5-hydroxy saxagliptin plasma concentration-time profiles in healthy subjects following a single oral dose of 2.5 mg saxagliptin ($n = 19$).

4. Conclusions

A simple and robust LC–MS/MS method for the determination of saxagliptin and 5-hydroxy saxagliptin in human K_2EDTA plasma was developed and validated for clinical studies. The procedure is straight-forward and involves a one step protein precipitation extraction combined with LC–MS/MS detection. The assay has been used to quantify saxaglipin and 5-hydroxy saxagliptin concentrations in human K_2EDTA plasma following PO administration. This method has determined to be specific, accurate and precise.

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