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ORIGINAL RESEARCH

Sensitive liquid chromatography tandem mass spectrometry method for the quantification of sitagliptin, a DPP-4 inhibitor, in human plasma using liquid–liquid extraction

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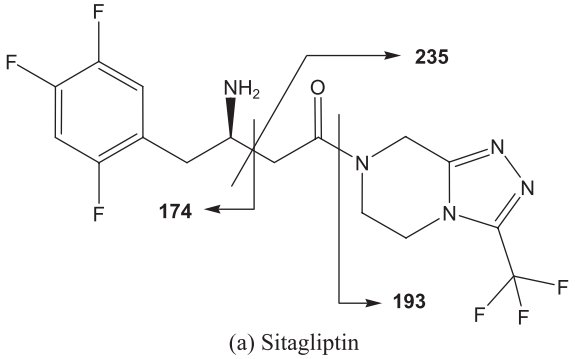
ABSTRACT: A sensitive high-performance liquid chromatography–positive ion electrospray tandem mass spectrometry method was developed and validated for the quantification of sitagliptin, a DPP-4 inhibitor, in human plasma. Following liquid–liquid extraction, the analytes were separated using an isocratic mobile phase on a reverse-phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective $[M + H]^+$ ions, *m/z* 408–235 for sitagliptin and *m/z* 310–148 for the internal standard. The assay exhibited a linear dynamic range of 0.1–250 ng/mL for sitagliptin in human plasma. The lower limit of quantification was 0.1 ng/mL with a relative standard deviation of less than 6%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. A run time of 2.0 min for each sample made it possible to analyze more than 300 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic studies. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: sitagliptin; DPP-4 inhibitor; liquid chromatography–tandem mass spectrometry; human plasma; pharmacokinetic study

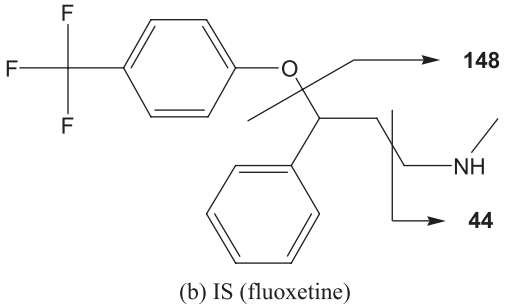
INTRODUCTION

Sitagliptin (Fig. 1), a dipeptidyl peptidase inhibitor (DPP-4 inhibitor), has recently been approved in the USA and Europe for the therapy of type 2 diabetes (Gallwitz, 2007; Lyseng-Williamson, 2007). DPP-4 inhibitors represent a new therapeutic approach to the treatment of type 2 diabetes that functions to stimulate glucose-dependent insulin release and reduce glucagons levels, by inhibiting the inactivation of incretins, particularly glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), thereby improving glycemic control (Herman *et al.*, 2007). Sitagliptin is effective in lowering HbA1c and fasting as well as postprandial glucose in monotherapy and in combination with other oral antidiabetic agents.

Sitagliptin is relatively rapidly absorbed (median t_{max} 1–4 h) after oral administration with an apparent terminal $t_{1/2}$ of approximately 8–14 h (Herman *et al.*, 2005).



(a) Sitagliptin



(b) IS (fluoxetine)

Figure 1. Chemical structures for (a) sitagliptin and (b) IS (fluoxetine).

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Abbreviations used: GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1.

Sitagliptin has a bioavailability of 87%, exhibits low and reversible binding to plasma proteins (approximately 38%), and is widely distributed in tissues (volume of distribution of 198 L) (Bergman *et al.*, 2007; Vincent *et al.*, 2007). The metabolism of sitagliptin represents a minor pathway of elimination and the majority of sitagliptin (79%) is excreted unchanged in urine (Herman *et al.*, 2007).

Pharmacokinetic applications require sensitive and selective assays with high sample throughput capacity. Recently Zeng *et al.* (2006) reported a method using high turbulence liquid chromatography (HTLC) online extraction with tandem mass spectrometry (MS/MS) for the quantification of sitagliptin in human plasma with a lower limit of quantitation of 0.5 ng/mL. A narrow bore large particle size reversed-phase column (Cyclone, 50×1.0 mm, 60 μm) and a BDS Hypersil C18 column (30×2.1 mm, 3 μm) were used as extraction and analytical columns, respectively. The retention times for sitagliptin and IS were about 2.8 and 2.9 min, respectively and the total run time was 5 min with gradient elution.

Liquid–liquid extraction can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS analyses. The present method consisted of a liquid–liquid extraction of sitagliptin and fluoxetin (commercially available internal standard) from 500 μL human plasma with tert-butyl methyl ether–dichloromethane (80:20, v/v). After extraction, the samples were injected onto a Waters Symmetry C_{18} reverse-phase chromatographic column for separation. The analyte was detected by tandem mass spectrometry using positive electrospray ionization in multiple reaction monitoring mode. The concentration of the analyte was calculated by peak area ratios of the analyte to the internal standard using standard curves generated with weighted linear regression analysis. The run time of the present method is 2.0 min, which insures high throughput. The method was validated over the concentration range 0.1–250 ng/mL. With dilution integrity up to 10-fold, we have established that the upper limit of quantification is extendable up to 2000 ng/mL. The method can be applied to generate pharmacokinetic data following the administration of sitagliptin in single and multiple ascending dose studies in human subjects.

EXPERIMENTAL

Chemicals. Sitagliptin and fluoxetine drug substances were obtained from the R&D department of Suven Life Sciences Ltd (Hyderabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv acetonitrile and HPLC-grade LiChrosolv methanol were purchased from Merck (Darmstadt, Germany). Tert-butyl methyl ether, dichloromethane and formic acid were purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC-MS/MS instrument and conditions. The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with an LC-AD VP binary pump, a DGU20A5 degasser and an SIL-HTC auto sampler equipped with a CTO-10AS VP thermostated column oven. The chromatography was performed using Symmetry C18, 150×4.6 mm, 5 μm at 30°C temperature. The isocratic mobile phase composition was a mixture of 0.03% formic acid–acetonitrile (30:70, v/v), which was pumped at a flow-rate of 1.0 mL/min with a split ratio of load to waste of 10:90.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada) using MRM. A turboionspray interface operating in positive ionization mode was used. Typical source conditions were as follows: the turbo-gas temperature was set at 250°C, and the ion spray needle voltage was adjusted at 5500 V. The mass spectrometer was operated at unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. The precursor/product ion pairs monitored were *m/z* 408–235 for sitagliptin and *m/z* 310–148 for the IS. Ion source gases 1 and 2 were set at 25 and 30 (arbitrary units) respectively; the curtain gas was at 15 (arbitrary units) and the collision gas at 10 (arbitrary units). The collision energy was set at 26 for sitagliptin and 12 for IS, respectively. Data acquisition was performed with analyst 1.4.1 software (MDS-SCIEX, Concord, Ontario, Canada).

Sample preparation. Standard stock solutions of sitagliptin (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (0.5 μg/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain sitagliptin concentration levels of 0.1, 0.2, 0.5, 1, 5, 10, 50, 100 and 250 ng/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 0.1 (lower limit of quantitation, LLOQ), 0.3 (low), 100 (medium) and 200 ng/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at below –50°C until analysis.

A plasma sample (0.5 mL) was pipetted into a 15 mL glass tube, then 25 μL of IS working solution (0.5 μg/mL) and 0.1 mL of 0.1 M sodium hydroxide were added. After vortex mixing for 10 s, 4 mL aliquot of the extraction solvent, tert-butyl methyl ether:dichloromethane (80:20, v/v), was added and the sample was vortex-mixed for 5 min. The organic layer (3 mL) was transferred to a glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μL of mobile phase and a 10 μL aliquot was injected into the chromatographic system.

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Bioanalytical method validation. A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and nine non-zero samples covering the total range 0.1–250 ng/mL, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing four sets of QC samples (LLOQ, low, medium and high concentrations) each comprising six replicates in a batch. The between-batch precision and accuracy were determined by analyzing five such different batches. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracies were 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations.

Recovery of sitagliptin from the extraction procedure was determined by a comparison of the peak area of sitagliptin in spiked plasma samples (six each of low, medium and high QCs) with the peak area of sitagliptin in samples prepared by spiking extracted drug-free plasma samples with the same amounts of sitagliptin at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples ($n = 6$) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (–50°C) and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels (0.3 and 200 ng/mL as low and high values) with six replicates for each.

RESULTS AND DISCUSSION

Mass spectrometry

In order to develop a method with the desired LLOQ (0.1 ng/mL), it was necessary to use MS/MS detection, as MS/MS methods provide improved limit of detection and selectivity for trace-mixture analysis. The inherent selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. $[M + H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The product ion mass spectra, and their proposed rationalizations in terms of fragmentation patterns of sitagliptin and IS, are illustrated in Fig. 2. The product ion mass spectrum of sitagliptin showed predominant fragment ions at *m/z* 235, 193 and 174. The ions at *m/z* 235 and 193 were allocated to the triazolopiperazine moiety, whereas the ion at *m/z* 174 was allocated to the trifluorophenyl-containing portion of the molecule. A minor fragment was observed at *m/z* 391, attributed to the loss of ammonia (NH_3). All product ions from portions of the molecule containing the primary amino group had a characteristic loss of a 17-amu fragment corresponding to the mass of ammonia. The product ion mass spectrum of the IS showed the formation of characteristic product ions at *m/z* 148 and 44. The most sensitive mass transition was from *m/z* 408 to 235 for sitagliptin and *m/z* 310 to 148 for the IS.

Method development

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. Six organic solvents, *n*-hexane, ethyl acetate, diethyl ether, dichloromethane, chloroform, *t*-butylmethylether and their mixtures in different combinations and ratios were evaluated. Finally, combination of tert-butyl methyl ether and dichloromethane (80:20 v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of sitagliptin from spiked plasma samples was 68.1 ± 1.1% and the recovery of the IS was 54.1 ± 1.0% at the concentration used in the assay (0.5 μg/mL). Recoveries of the analytes and IS were good and it was consistent, precise and reproducible. The assay has proved to be robust in high-throughput bioanalysis.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several attempts to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 0.03% formic acid–acetonitrile (30:70, v/v) could achieve this purpose and was finally adopted as the mobile phase. The high proportion of organic solvent eluted both the analyte and IS at retention time of 0.94 min. A flow rate of 1.0 mL/min produced good peak shapes and permitted a run time of 2.0 min.

The pH of the aqueous phase of the liquid chromatographic mobile phase influences both the chromatographic elution of the compounds and the formation of the $[M + H]^+$ molecular ions and is strongly related to their degree of ionization. The pK_a values of the analyte and IS were calculated using the MarvinSketch/Swing 4.0.3 software. As both sitagliptin and fluoxetine are basic compounds with pK_a values 8.8 and 9.8, respectively, the use of slightly acidic solutions favors ionization of the analytes by protonation of their basic sites. Therefore, it was found that positive ionization of

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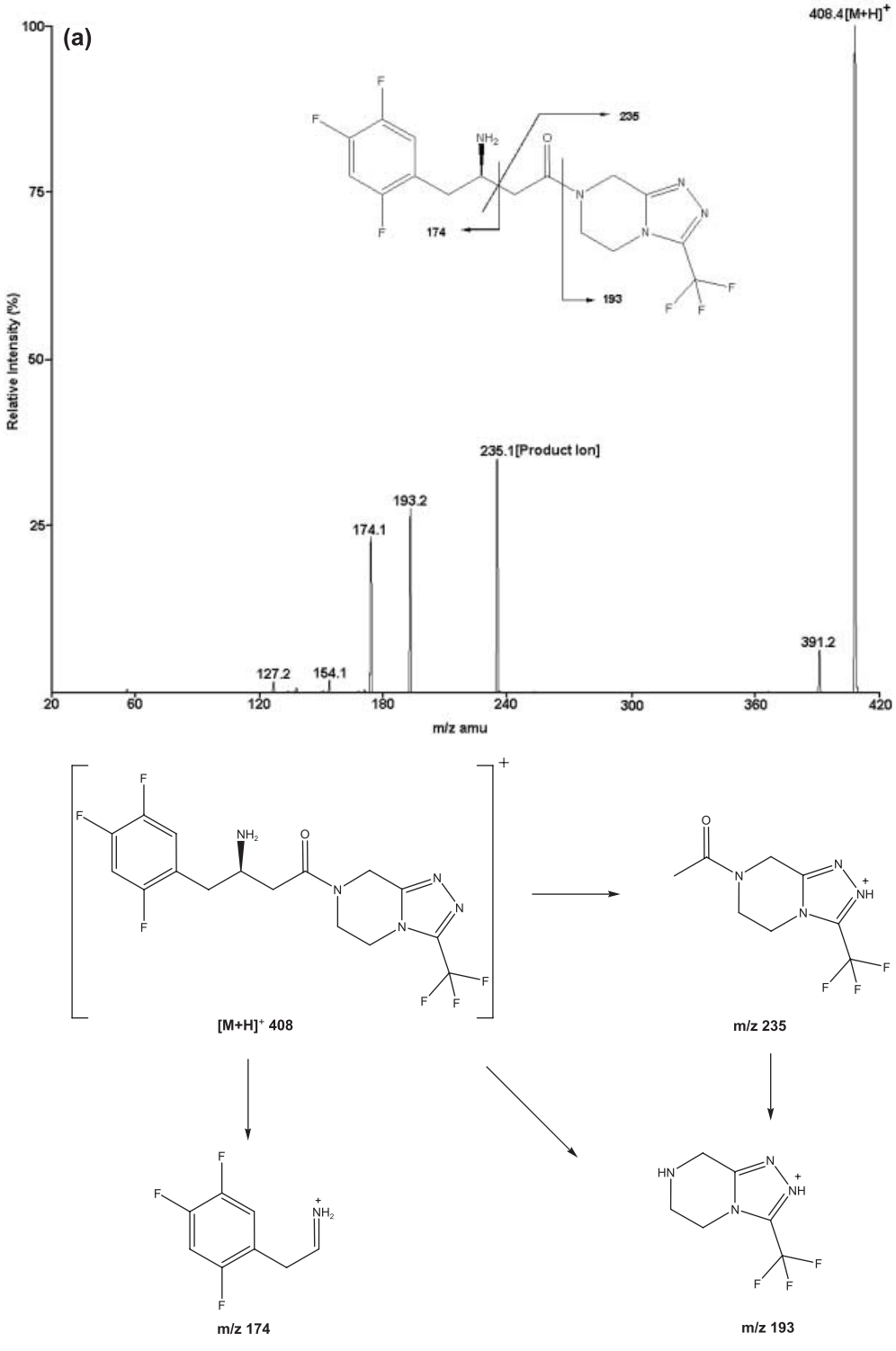

Figure 2. Full-scan positive ion turboionspray product ion mass spectra and the proposed patterns of fragmentation of (a) sitagliptin and (b) fluoxetine (internal standard).

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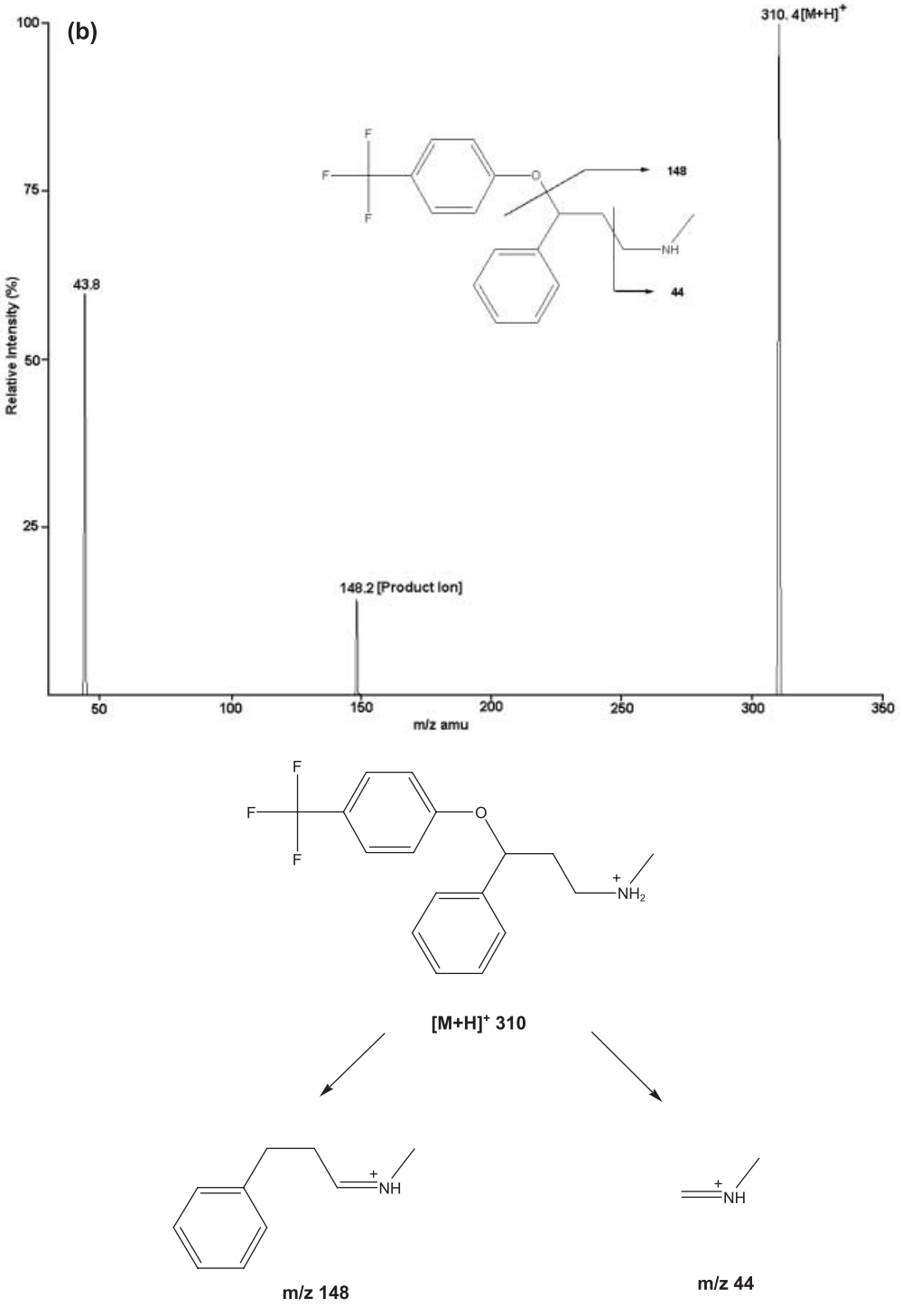

Figure 2. (*Continued*).

the compounds in the electrospray in source increases in acidic mobile phases.

Choosing the appropriate internal standard is an important aspect to achieving acceptable method performance, especially with LC-MS/MS, where matrix effects can lead to poor analytical results. Ideally, an isotopically labeled internal standard for the analyte should be used, but it is not commercially available. Several compounds were investigated to find a suitable IS, and finally fluoxetine was found to be suitable. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components from the sample matrix. The importance of including the evaluation of matrix effect

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Table 1. Precision and accuracy of the method for determining sitagliptin concentrations in plasma samples

	Within-batch (n = 3)			Between-batch (n = 5)		
Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)
0.1	0.10 ± 0.01	12.9	97.7	0.10 ± 0.01	5.1	99.0
0.3	0.29 ± 0.02	6.6	95.4	0.28 ± 0.01	3.9	93.6
100	102.50 ± 6.28	6.1	102.5	98.51 ± 6.55	6.7	98.5
200	193.15 ± 15.01	7.8	96.6	191.19 ± 10.33	5.4	95.6
2000[a]	1996.37 ± 123.64	6.2	99.8	1984.28 ± 98.61	5.0	99.2

[a] The sample was processed with 10-fold dilution.

in any LC-MS/MS method is outlined in an excellent paper by Matuszewski *et al.* (2003). Their data strongly emphasize the need to use a blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore all validation experiments in this assay were performed with matrixes obtained from different individuals. In addition, validation experiments were performed using hemolytic and strongly lipemic matrixes. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

Assay performance and validation

The nine-point calibration curve was linear over the concentration range 0.1–250 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of $y = 0.8572(\pm 0.0000)x + 0.8577(\pm 0.0007)$ where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9979 ± 0.009.

The selectivity of the method was examined by analyzing (n = 6) blank human plasma extract [Fig. 3(A)] and an extract spiked only with the IS [Fig. 3(B)]. As shown in Fig. 3(A), no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Fig. 3(B) shows the absence of direct interference from the IS to the MRM channel of the analyte. Figure 3(C) depicts a representative ion-chromatogram for the LLOQ (0.1 ng/mL). Excellent sensitivity was observed for a 10 µL injection volume; the LLOQ corresponds to ca. 3 pg on-column.

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision and was found to be 0.1 ng/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (0.1 ng/mL) was ~10-fold greater than the mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The between-batch precision at the LLOQ was 5.1%, and the between-batch accuracy was 99.0% (Table 1). The within-batch precision was 12.9% and the accuracy was 97.7 for sitagliptin.

The lower and upper quantification levels of sitagliptin ranged from 0.3 to 200 ng/mL in human plasma. For the between-batch experiments the precision ranged from 3.9 to 6.7% and the accuracy from 93.6 to 98.5% (Table 1). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria (<±15%).

The upper concentration limits can be extended with acceptable precision and accuracy to 2000 ng/mL by a 10-fold dilution with control human plasma. These results suggested that samples with concentrations greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data (Table 1).

Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 22 h). Samples were extracted and analyzed as described above and the results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 22 h was also assessed. The results indicate that solutions of the analyte and the IS can remain in the autosampler for at least 22 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The stability data of the analyte in plasma over three freeze–thaw cycles indicate that the analyte is stable in human plasma for three freeze–thaw cycles, when stored at below –50°C and thawed to room temperature.

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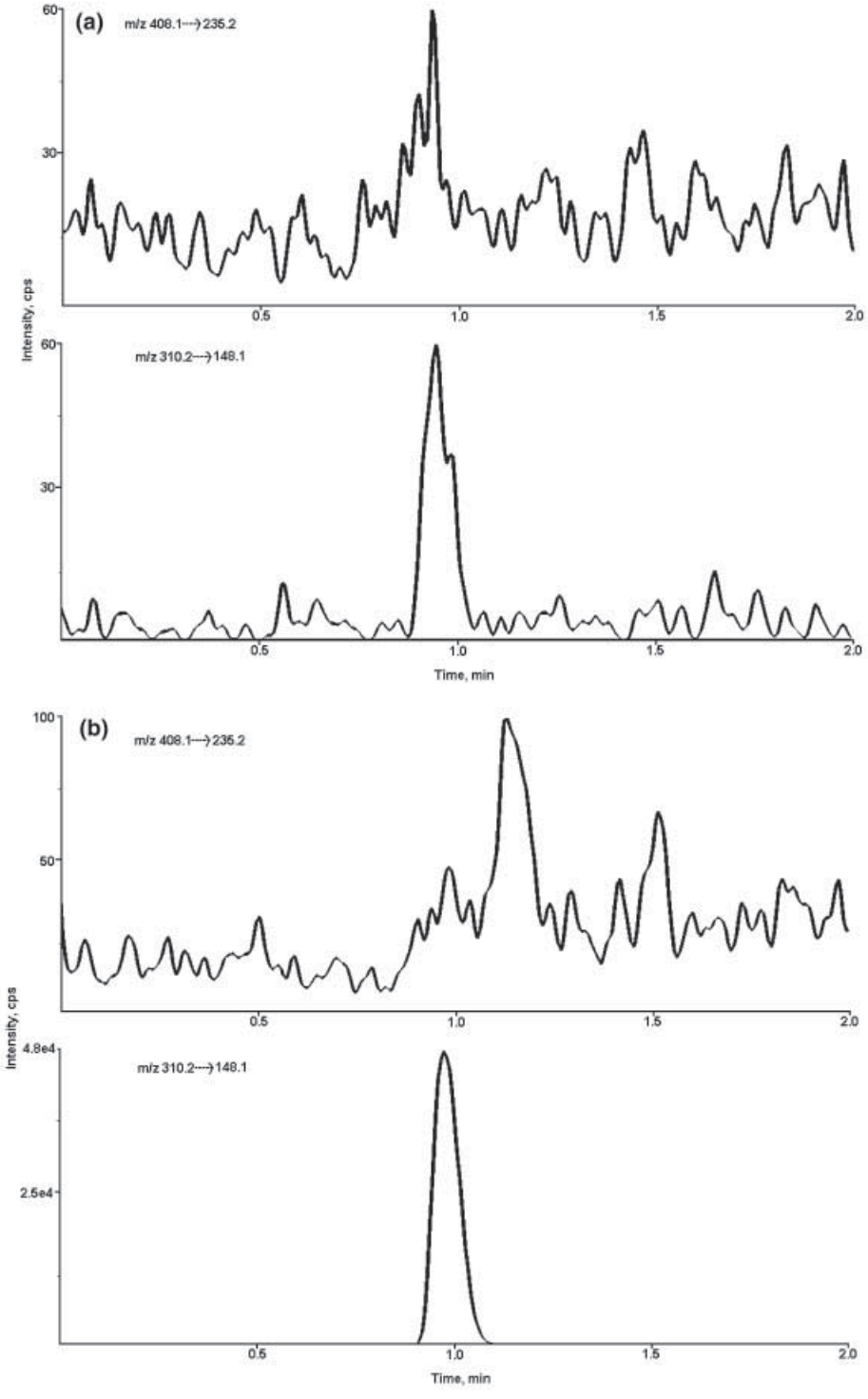

Figure 3. MRM chromatograms for sitagliptin and IS resulting from analysis of: (a) blank (drug and IS free) human plasma; (b) zero sample (drug-free spiked with IS) human plasma; (c) 0.1 ng/mL (LLOQ) of sitagliptin spiked with the IS.

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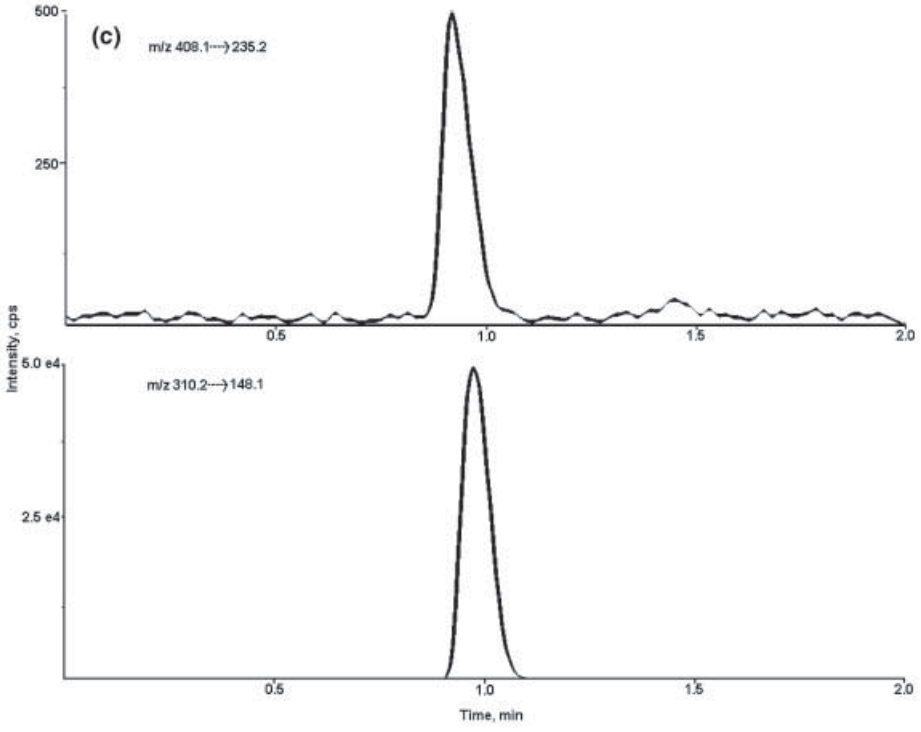

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The long-term stability data of the analyte in human plasma stored for a period of 30 days at below –50°C showed reliable stability behavior, as the mean results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of the analyte in plasma samples at below –50°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic studies.

The stability of the stock solutions was tested and established at room temperature for 6 and 22 h, and under refrigeration (4°C) for 30 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application

The method was applied to determine the plasma concentrations of sitagliptin following a single 100 mg oral administration to 12 healthy subjects. The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a pharmacokinetic study are depicted in Fig. 4.

CONCLUSIONS

In summary, a method is described for the quantification of sitagliptin in human plasma by LC-MS/MS using liquid–liquid extraction and fully validated according to commonly accepted criteria. The current method has shown acceptable precision and adequate sensitivity for the quantification of sitagliptin in human plasma samples obtained for pharmacokinetic studies. The desired sensitivity of sitagliptin was achieved with an LLOQ of 0.1 ng/mL, which has a within- and between-batch CV of 12.9 and 5.1% respectively. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results.

The method demonstrated has broader calibration range with acceptable accuracy and precision. With dilution integrity up to 10-fold, we have established that the upper limit of quantitation is extendable up to 2000 ng/mL. Hence, this method is useful for single and multiple ascending dose studies in human subjects. The simplicity of the assay and using rapid liquid–liquid extraction and sample turnover rate of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of sitagliptin. The method was successfully applied to quantify the concentrations of sitagliptin in a clinical pharmacokinetic study.

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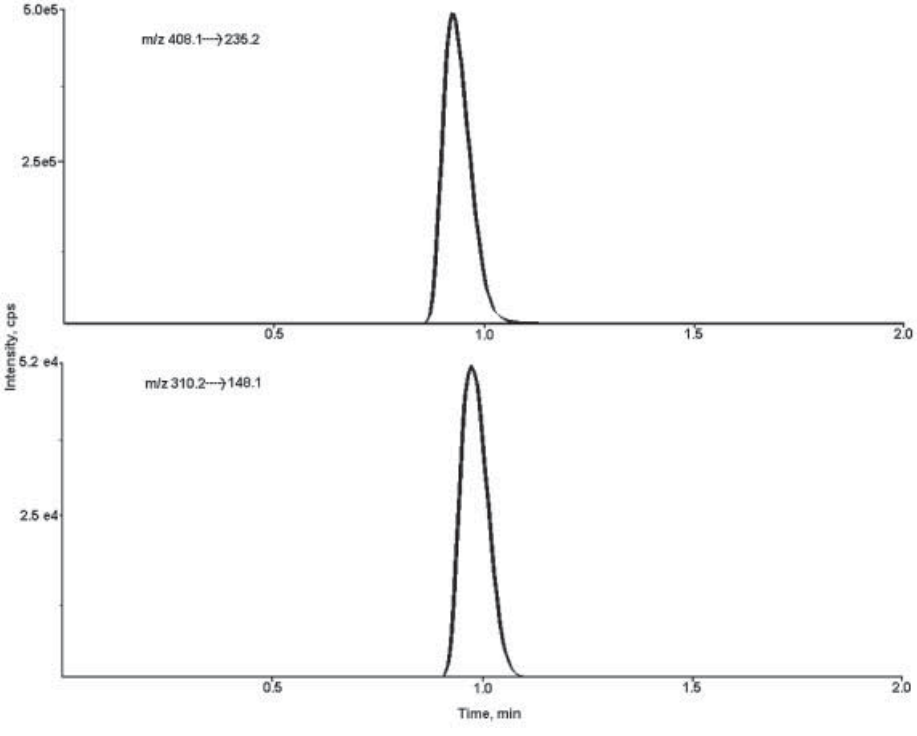

Figure 4. MRM chromatograms resulting from the analysis of a subject plasma sample after the administration of a 100 mg oral single dose of sitagliptin.

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