# Liquid chromatography–electro spray ionization–tandem mass spectroscopy method for the quantification of alogliptin in spiked human plasma

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**Background**
Alogliptin is the inhibitor of dipeptidyl peptidase-4. It acts to regulate blood sugar by increasing the amount of insulin in the body.
**Aim**
A liquid chromatography–mass spectroscopy/mass spectroscopy method was developed for the estimation of alogliptin in spiked human plasma. Liquid–liquid extraction technique was adopted for the extraction of alogliptin from human plasma.
**Materials and methods**
The chromatographic separation was performed on a Waters Symmetry shield RP C-18 column with 4.6-mm internal diameter, 5-μm particle size, 100A° pore size analytical column at a flow rate of 1.0 ml/min using a mixture of 0.3% formic acid and acetonitrile in the ratio of 20 : 80% v/v as mobile phase. Positive ion mode was selected to obtain the product ion m/z +339.19 (parent) →245.11 (product) for alogliptin and m/z +303.39 (parent) →232.16 (product) for internal standard.
**Results**
The developed method was satisfactorily validated as per United State Food and Drug Administration guidelines for the bioanalytical study because it exhibits excellent intraday and interday accuracy with % nominal 91.06→98.48% and precision percentage coefficient variation less than or equal to 2% in all quality control levels. Alogliptin revealed its linearity with correlation coefficient ($r^2$=0.99) in the concentration range of 40.17–16 096 ng/ml, showed acceptable % extraction recovery (96.18→98.36%), and showed excellent matrix and analyte selectivity (% interference=0), as well as matrix effect (matrix factor 0.931 at lower quantitation limit and 1.14 at high quality control level). The stability study results of bench top, freeze thaw, autosampler, and short-term and long-term showed the accuracy in the range of 92.52–99.16% and percentage coefficient variation in the range of 0.22–1.93.
**Conclusion**
The method was successfully optimized and validated as per the United State Food and Drug Administration bioanalytical method development guidelines. The applicability of the developed method undoubtedly can further extend during preclinical and clinical trials.

**Keywords:**
alogliptin, bioanalytical, human plasma, validation

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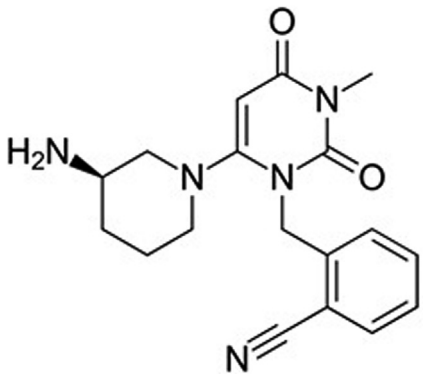
## Introduction

Alogliptin belongs to a class of drugs known as dipeptidyl peptidase-4 inhibitors. By raising the amount of insulin in the body, it serves to control blood sugar. Chemically speaking, alogliptin is 2-({6-[(3R)-3-aminopiperidin-1-yl]-3methyl-2, 4-dioxo-3, 4-dihydropyrimidine-1 (2H)-yl}methyl) benzonitrile (Fig. 1) and belongs to the class of antidiabetic drugs administered by mouth. Alogliptin is used to reduce blood sugar levels in patients with type 2 diabetes along with diet and exercise. In several countries around the world, the dipeptidyl peptidase-4 alogliptin inhibitor is approved for the treatment of type 2 diabetes mellitus [1,2]. It is metabolized to two minor metabolites as an N-demethylated metabolite and an N-acetylated metabolite. M-1 and M-2 are formed and represent less than 2 and 6%, respectively, of parent drug concentrations in plasma and urine [3].

Fixed-dose alogliptin/metformin and alogliptin/pioglitazone formulations are also available. A systematic literature review was undertaken, and several methods were discovered to be documented using ultraviolet–visible spectroscopy [4], the high-performance liquid chromatography (HPLC)

**Figure 1**



Chemical structure of alogliptin.

reverse-phase alogliptin estimation technique alone, and in combination [5–9]. Estimates of alogliptin in human plasma using liquid chromatography–mass spectroscopy (LC–MS) in conjunction with other antidiabetic drugs, such as metformin, pioglitazone, and voglibose, have also been published [10–13]. These assay methods have many important disadvantages, such as low sensitivity, difficulty of the procedure, higher volume of sample requirements, time consuming, and expensive, limiting the omnipresent acceptability of clinical trials and other study. No method has been published yet for estimation of alogliptin alone in human plasma. Alogliptin alone is also generally used to treat diabetes. A method of estimation of alogliptin alone in human plasma using the LC–MS/MS technique is therefore required. The use of vildagliptin as an internal standard (IS), a fast method was established here. The used liquid chromatographic system used new-generation columns, that is packed with superficially porous silica particles; these provide ultrafast HPLC separations for a variety of compounds with moderate sample loading capacity and low back pressure [14–16]. Analysis at trace levels, an ideal area of application for hyphenated techniques, is steadily gaining importance. Many sample preconcentration and clean-up methods have been hyphenated with core analytical techniques to accomplish the task of low-level detection [17,18].

The developed method shows high-throughput results than the few reported LC–MS/MS methods as the requirement of total analysis time is very less with less run time. An easy liquid–liquid plasma extraction procedure shows this method more practically easy and cost-effective. The present established method has been validated according to the specification of the United State Food and Drug Administration (USFDA) bioanalytical method development and validation [19] to further implement this approach during clinical and preclinical trials for further bioequivalence and toxicological studies.

## Materials and methods

### Instrumentation

The authors attempted to establish a liquid chromatographic system for the determination of alogliptin using Mass Lynx V 4.1 program using a Quattro Premier XE LC–MS/MS (Waters Corporation 34 Maple St, Millford, MA, USA)) coupled with 2695 HPLC separation module, a water symmetry shield RP C-18 column with 4.6-mm internal diameter, 5-µm particle size, column, and an SPD-M20A PDA detector (7102 Riverwood Drive, Columbia, Maryland, USA). Data acquisition was done by using Mass Lynx V 4.1 software.

### Analyte and internal standard

The reference samples of alogliptin and vildagliptin (IS) were procured from M/s Hetero Drug Ltd, (Hyderabad, India).

### High-performance liquid chromatographic operating conditions

Mixtures of widely used solvents such as water, methanol, and acetonitrile with or without buffers in different combinations were tested as mobile phases on Waters Symmetry shield RP C-18 column with 4.6-mm internal diameter, 5-µm particle size, and 100A° pore size. A binary mixture of 0.3% formic acid (3.0 ml of formic acid in 1000 ml) and acetonitrile in the ratio of 20 : 80 percent v/v for the ideal separation of the drug under isocratic conditions was proven to be the most acceptable of all combinations, as the chromatographic peaks obtained were well described and resolved and tailing-free. A mobile phase flow rate of 1.0 ml/min was found to be suitable in the study range of 0.8–1.5 ml/min. Detection of the ions was carried out in multiple reaction monitoring by monitoring the transition m/z +339.19 (parent)→ 245.11 (product) for alogliptin and m/z +303.39 (parent)→232.16 (product) for IS. The 10 µl of the prepared sample solution was injected into the LC–MS/MS system. Under these conditions, the alogliptin and IS were eluted within 2.186 and 2.314 min, respectively.

### Mass spectrometry operating conditions

The analyte molecule was tuned by injection of 0.5 µg/ml concentration on the MS to detect the parent ion and the daughter ion. Tuning was carried out in both positive and negative ionization modes, but the positive polarity mode demonstrated better sensitivity with

**Table 1 Tuning parameters of alogliptin and vildagliptin**

| ES+source parameter | Settings | Analyzer parameter | Settings |
|---|---|---|---|
| Capillary (kV) | 3.00 | LM resolution 1 | 13.0 |
| Cone (V) | 22 | HM resolution 1 | 13.0 |
| Extractor (V) | 5 | Ion energy 1 | 0.5 |
| RF Lens (V) | 0.0 | Entrance | −1 |
| Source temp (0°C) | 100 | Collision | 20 |
| Desolvation temp (0°C) | 450 | Exit | 0 |
| Cone flow (l/h) | 50 | LM resolution 2 | 13.0 |
| Desolvation flow (l/h) | 800 | HM resolution 2 | 13.0 |

greater reproducibility. In Table 1, all of the optimized potential parameters in the positive mode are given.

#### Preparation of CC stock solution

Alogliptin standard equivalent to 10.0 mg of alogliptin was weighed and transferred into a 5-ml volumetric flask. It was dissolved in methanol and made up the volume using the same solvent to achieve 2 mg/ml. It was mixed well, and the solution was stored at 2–8°C.

#### Preparation of CC spiking solutions and spiked calibration curve plasma standards

Using human plasma as the diluent, calibration curve dilutions were prepared from alogliptin stock solution in the concentration range of 1996.800–800 000 000 ng/ml. These dilutions (CC spiking solutions) were subsequently used to spike the blank plasma that was screened. The solution was stored at 2–8°C. It was blended well. To spike the screened blank human plasma matrix, the above calibration curve dilutions (CC spiking solutions) were used to prepare the plasma calibration curve standards ranging from 40.176 to 16 096 ng/ml. Storage temperature was maintained at approximately −20°C. In prelabeled polypropylene vials, aliquots of 0.3 ml of the aforementioned plasma calibration curve requirements were taken, which were then tightly capped and deposited at −20°C in a freezer.

#### Preparation of QC stock solution

Alogliptin standard equivalent to 15.0 mg of alogliptin was weighed and transferred into a 5-ml volumetric flask. It was dissolved in methanol and made up the volume using the same solvent to achieve 3.0 mg/ml. It was mixed well, and the solution was stored at 2–8°C.

#### Preparation of QC spiking solution and spiked QC plasma samples

The quality control dilutions (QC spiking solutions) from alogliptin stock solution were prepared in the concentration range from 2012.511 to 6 80 400.000 ng/ml using human plasma as the diluent. The serial dilutions (QC spiking solutions) were subsequently used for spiking the screened blank plasma. The aforementioned quality control dilutions (QC spiking solutions) were used to spike the screened blank human plasma to prepare the plasma quality control samples ranging from 40.250 to 13 608.000 ng/ml. In these ranges of sample, various quality control samples were considered as 13 608 ng/ml [high quality control (HQC)], 7620.48 ng/ml [medium quality control (MQC-1)], 823.01 ng/ml (MQC-2), 117.69 ng/ml [lower quantitation limit (LQC)], and 40.25 ng/ml [lower limit of quantitation (LLOQ)]. Overall, 0.3 ml of aliquot of the above plasma quality control samples was taken in prelabeled polypropylene vials which were then capped properly and stored in a freezer at −20°C.

#### Sample preparation and extraction procedure

Required number of plasma samples were taken from the deep freezer, thawed at room temperature, and then vortexing of the tubes was done to mix. Then, 200 µl of plasma was transferred into prelabeled tubes. Then, 50.0 µl of 3.0 µg/ml ISTD working solution was added to all the vials excepting the STD blank and vortexed for about 15 s. Overall, 100.0 µl of extraction buffer was added to all the vials and vortexed for about 30 s. Then, 2.5 ml of ethyl acetate was added to the all vials and vortexed for a period of 20 min, in roto spin at 40 rpm. All the vials were centrifuged at 4500 rpm, at 4°C for 5 min. Approximately 2.0 ml of supernatant was transferred into prelabeled tubes, and the supernatant solutions were evaporated to dryness under nitrogen at 40±5°C. Overall, 400.0 µl of reconstitution solution was added to all the tubes and vortexed for about 30 s. Appropriate volumes of the reconstituted solutions were transferred into prelabeled autosampler vials, and 10 µl was injected into LC–MS/MS system to obtained the multiple reaction monitoring chromatograms.

#### Method validation

The validation parameters have been studied to justify the developed method as per the USFDA bioanalytical method development guidelines.

#### Specificity and selectivity

At least six dissimilar lots of $K_2$ EDTA blank human plasma samples were analyzed to examine the interferences at the retention time of analytes and IS. The interference at the retention times of the drugs by comparing the response in the blank plasma against the response of LLOQ was evaluated. Moreover, the interference at the retention time of IS was also evaluated against the response of the extracted IS in LLOQ sample. The response of the interfering substance will be acceptable if it is less than 20% of

the mean drug response in LLOQ sample and less than 5% in case of IS.

#### Carryover effect

It was done in various parts of the system to verify the effect of carrying analytes and the internal norm. Six blank matrix replicates (extracted) and six high-concentration replicates (extracted) of analytes at the stage of the calibration curve range as (HQC), six extracted LLOQ concentrations of the analytes and IS were prepared and injected as per sequence; the sequence is, first six injections was extracted LLOQ, followed by extracted HQC and extracted blank plasma. HQC and blank plasma samples were injected alternatively. Interfering peaks response in blank samples at the retention time of the analytes must be less than or equal to 20% of average drug response and for IS must be less than or equal to 5% of average IS response in LLOQ samples.

#### Recovery

The extraction efficiency of human plasma analytes and IS was calculated by comparing the analytes extracted from replicate QC samples (n=6) responses with that of neat standard solutions that were spiked at equivalent concentrations extracted by the method of protein precipitation in the postextracted human plasma blank sample. Single concentration was used to determine recovery of IS, whereas in case of analytes (alogliptin), the recovery was determined at LQC as well as HQC concentrations. Mean overall % recovery was calculated, and % of difference should not be more than 25% between highest and lowest % recovery.

#### Matrix effect

Matrix effect was determined by assessing the effect of matrix that is, the human plasma constituents over the ionization of analytes and IS by following postcolumn infusion method. A constant amount of analyte into LC system outlet entering to MS inlet is delivered by the infusion pump. The MS was operated in multiple reaction monitoring mode. Sample extracts were injected on LC column under similar chromatographic condition. A steady ion response was obtained as a function of time. Any endogenous compound eluted from the column and causing a variation in electro spray ionization response of the infused analyte was perceived as a suppression or enhancement of infused analyte response. At a constant rate, the analytes and IS solutions were infused. Blank matrix sample was injected through the LC. Six different lots of human plasma samples were spiked with analyte concentration levels at concentrations of LQC and HQC levels to evaluate matrix effect in addition to postcolumn infusion method. The acceptance criteria were ±15% deviation from the nominal value for each back-calculated concentration.

#### Linearity

The linearity of the method was determined by using a $1/X^2$ weighted least square regression analysis of standard plots associated with a 10-point standard curve. All the three calibration curves analyzed were found to be linear for the standard concentration ranging from 40.176 to 16 096.225 ng/ml. The acceptance criteria for the linearity is the $r^2$ (coefficient of correlation) should be more than or equal to 0.98.

#### Precision

The precision [percentage coefficient variation (%CV)] of the LC–MS/MS method was evaluated by analyzing six replicates at different concentration levels corresponding to HQC, MQC-1, MQC-2, LQC, and LLOQ during the course of validation.

#### Accuracy

The accuracy of the assay was evaluated by analyzing six replicates at different concentration levels corresponding to HQC, MQC-1, MQC-2, LQC, and LLOQ during the course of validation. The accuracy was calculated as the absolute value of the ratio of mean values of the quality control samples to their respective nominal values, expressed as percentage. The acceptance criteria for accuracy (% nominal) is ±15 and ±20% only for LLOQ level and for precision (%CV) should be within ±15% and only for LLOQ sample it is ±20%.

#### Dilution integrity

To study the dilution integrity of the developedmethod dilution integrity spiking solution (6 130 863.637 ng/ml) which is approximately equivalent to 2.5 times of the highest CC spiking solution was utilized. Six sets of dilution integrity samples were diluted five times and another six samples were diluted to 10 times. Samples were analyzed and concentrations were calculated by multiplying dilution factors, 5 (for five times dilution) and 10 (for 10 times dilution). For each dilution level, at least four out of six of the QC samples should be 15% of their respective nominal.

#### Ruggedness

To carry out the ruggedness study of the developed method, one precision and accuracy batch of samples were processed and analyzed with different columns of the same make and with different reagent lots.

### Stability studies

A series of stability study (bench top, freeze thaw, autosampler, short-term stability, and long-term stability) of alogliptin was performed using calibration curve samples (freshly prepared) and quality control samples in all the levels and analyzed. Concentration response linearity data were collected and used to calculate the concentration of stability samples.

### Bench top stability

LQC and HQC samples of six sets were collected from the deep freezer and placed unprocessed for the period of 12 h. After that suitable period bench top stability samples were processed and analyzed along with fresh samples. Linearity data were utilized for the calculation of the amount.

### Autosampler stability

Six sets of quality control samples were prepared in LQC and HQC level and kept in autosampler for 3 days. After the suitable period, all the stability samples were quantified against the freshly prepared spiked calibration curve and quality control samples at low middle and high QC levels.

### Freeze thaw stability

It was determined by four freeze-thaw cycles. LQC and HQC samples of six replicates were prepared and stored in deep freezer at −70°C. After 24 h, first six samples were withdrawn and thawed at room temperature and refrozen again. Similarly, the remaining samples were also withdrawn after next 12 h, thawed, and refrozen again. All samples were processed after suitable four cycles. Stability samples were analyzed in LC–MS/MS system, and quantity was calculated from the freshly spiked calibration samples and quality control samples.

### Short-term stability

Quality control samples (six sets) at low and high level were prepared and stored at deep freezer at −20°C. After 3 days, on the day of evaluation, samples were processed along with freshly prepared quality control samples in all the levels and calibration curve samples. The concentration of the stability samples was calculated.

### Long-term stability

To conduct long-term stability study, LQC and HQC samples were prepared and kept for 30 days at −70°C. Six sets of long-term quality control samples (LQC and HQC) were withdrawn on the day of evaluation, thawed at room temperature, and processed. All stability samples were introduced into the LC–MS/MS system. All the stability samples were quantified from the calibration curve data obtained from the freshly prepared samples. For all stability samples, mean of % nominal at each quality control level should be between 85 and 115% and the precision must be less than or equal to 15% of the %CV. At least 67% of the stability QC samples should be within 15% of their respective nominal values.

## Results

### Mass spectrometry

MS parameters were correctly tuned for both the analyte and the internal norm in positive and negative ion modes. As an optimized state, the positive ion mode and multiple reaction mode were finalized. For both analytic and internal norms, different parameters of multiple reaction control have been optimized. Table 1 has been cited for the specifics of the tuned parameters. The mass spectra of alogliptin are shown in Fig. 2.

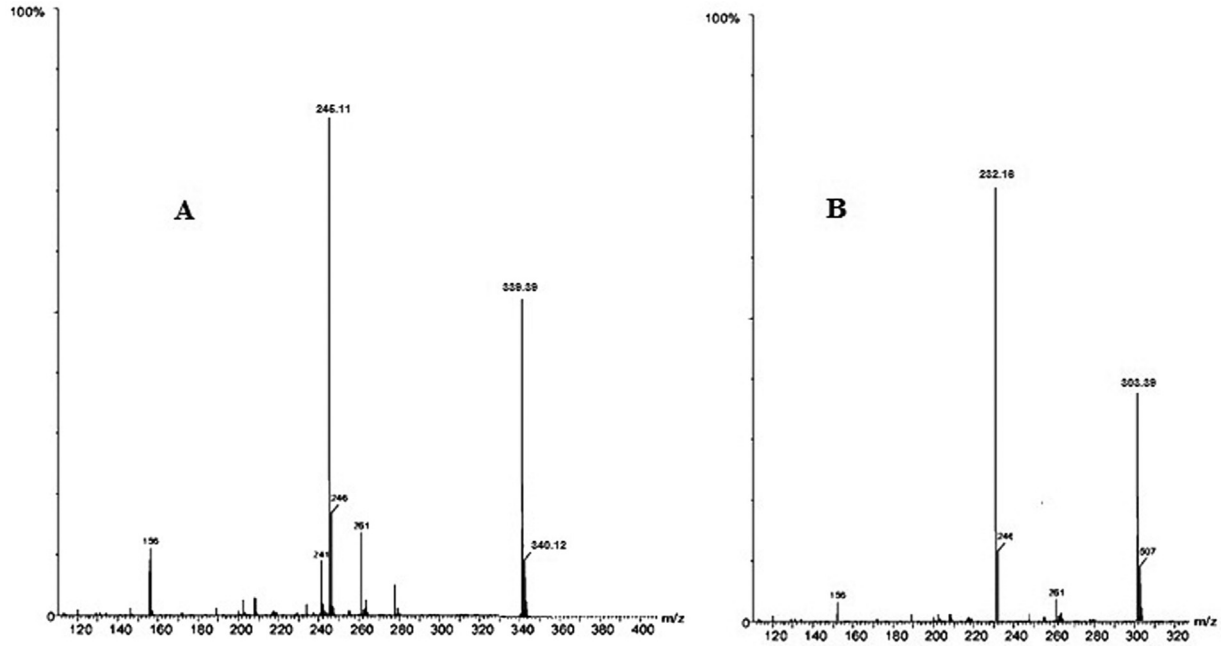
### Method development

Several chromatographic trials have been conducted by utilizing different mobile phases with several volume ratio of solvents and columns. Various combinations of acetonitrile and buffers have been used during initial trials; different types of columns like C-8, C-18 of hypersil, Zorbax, and kromasil have been used. Waters Symmetry shield RP C-18 column, with 4.6-mm internal diameter, 5-µm particle size, and a binary mixture of 0.3% formic acid (3.0 ml of formic acid solution into 1000 ml volumetric flask and made up the volume with Milli-Qwater) and acetonitrile in the ratio of 20 : 80% v/v, was proved to be the most suitable. Vildagliptin was selected as an IS because the retention time and other values were very selective and lack of interference with analytes. Under this optimized condition, the retention time of alogliptin was found to be 2.186 min, and for IS, 2.314 min was found, as shown in Fig. 3 for blank plasma sample and Fig. 4 for alogliptin and IS at various quality control levels.

### Validation

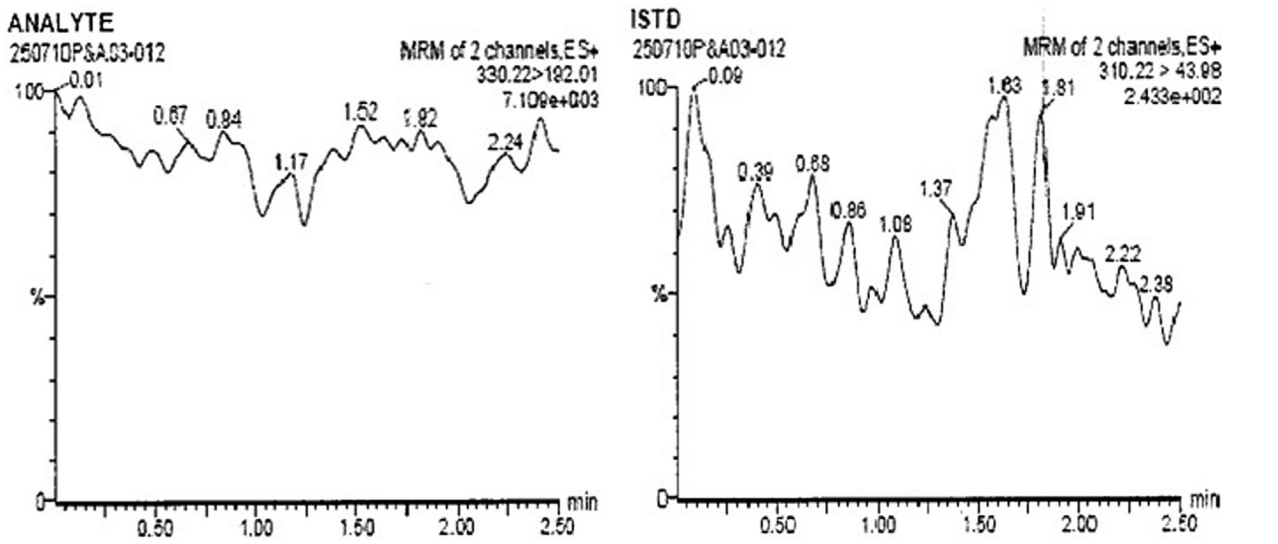
Various validation parameters have been studied using the optimized condition and using several quality control and calibration curve samples. In matrix selectivity study, no interference was observed at the retention time of analyte and IS when compared with blank matrix response against the mean response of extracted LLOQ samples. The response of interfering peaks at the retention time of analyte and IS was found 0% of the mean analyte response. In analyte selectivity study, no interference was observed at the retention time of analyte in the presence of IS. In the carryover test results, the obtained response for blank samples is 0

**Figure 2**




Product ion mass spectra of alogliptin (a) and internal standard vildagliptin (b).

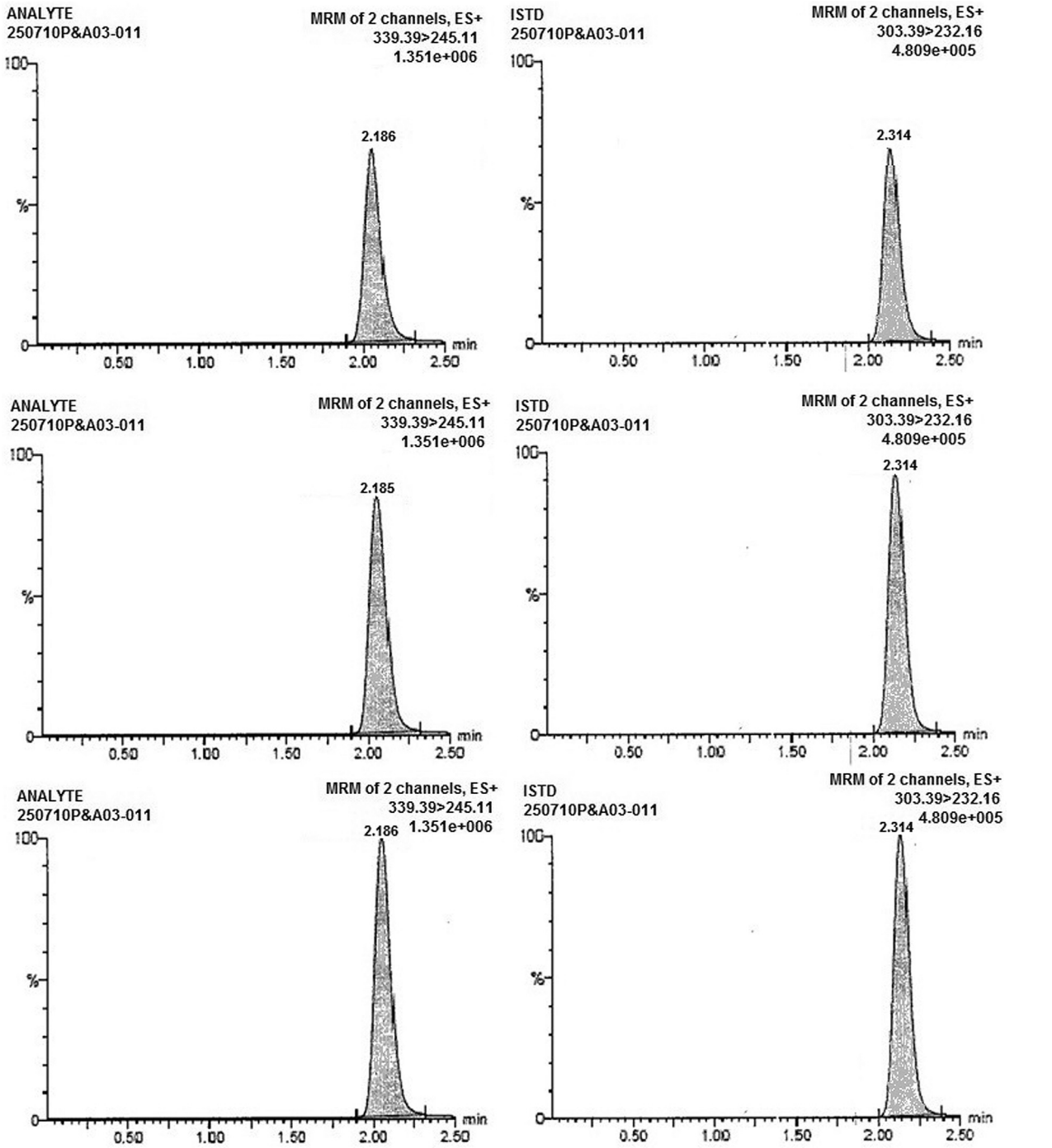
**Figure 3**




MRM chromatogram of blank plasma. MRM, multiple reaction monitoring.

and calculated % carryover is also 0%, which indicates no interference was observed at the retention time of the analyte and IS at ULOQ and LLOQ level. In the study of matrix effect, the IS normalized factor was calculated; the %CV of IS normalized factor was found 12.07% for LQC and 10.85% for HQC samples. Intraday within batch precision (%CV) for LLOQ, LQC, MQC-1, MQC-2, and HQC samples of alogliptin was found within 0.35–14.7. Intraday accuracy for LLOQ, LQC, MQC-1, MQC-2, and HQC was found in the range of 90.91–110.23%. Between batch accuracy (% nominal) results for the levels of LLOQ, LQC, MQC and HQC were between 95.95 and 100.52% and precision (%CV) values were between 5.93 and 14.35; details of the results are cited in Table 2. In the linearity study, the weighing factor, 1/(concentration ratio)$^2$ of analyte concentration to IS concentration, shows a correlation coefficient ($r^2$=0.99) in the concentration range of 40.17–16 096 ng/ml for alogliptin. The mean back-calculated concentrations for linearity with mean accuracy values have been accumulated and cited in Table 3

**Figure 4**




MRM chromatograms of alogliptin and internal standard at LQC level (a). MQC-1 level (b) and HQC level (c). HQC, high quality control; LQC, lower quantitation limit; MQC, medium quality control; MRM, multiple reaction monitoring.

and Fig. 5. In the dilution integrity study at ULOQ level was quantified and %CV and % nominal was found 2.08 and 98.81 at five times dilution and 2.20 and 95.33 at 10 times dilution in comparison with the undiluted calibration curve samples. The mean overall recovery of alogliptin in recovery study was found 88.63% with a precision (%CV) of 8.28. The mean recovery of IS is 93.15. In ruggedness study using different column the %CV (precision) was found to be 1.14, 1.41, 1.37, 4.61, and 16.34 for HQC, MQC-1, MQC-2, LQC, and LLOQ, respectively, whereas the accuracy was found to be 98.04, 101.84, 109.88, 108.60, and 87.87 for HQC, MQC-1, MQC-2, LQC, and LLOQ, respectively. The ruggedness study also conducted for different analyst, where %CV (precision) was found to be 1.51, 1.08, 0.51, 6.84, and 9.14 for HQC, MQC-1, MQC-2, LQC, and LLOQ, respectively, whereas the accuracy was found to be 97.74, 98.51, 101.85, 99.31, and 87.87 for HQC, MQC-1, MQC-2, LQC, and LLOQ, respectively.

**Table 2 Accuracy and precision for determination of alogliptin in spiked human plasma**

| | | Alogliptin measured concentration (ng/ml) | | | |
|---|---|---|---|---|---|
| QC levels | Run[a] | Mean | SD | %CV | % nominal |
| | | Between the batch intraday | | | |
| LLOQ (40.25 ng/ml) | 1 | 36.49 | 2.56 | 2.07 | 94.53 |
| | 2 | 38.77 | 4.53 | 1.46 | 98.07 |
| | 3 | 36.22 | 11.02 | 1.02 | 94.05 |
| | 4 | 36.01 | 4.02 | 0.05 | 94.77 |
| LQC (117.69 ng/ml) | 1 | 114.34 | 4.66 | 0.54 | 95.76 |
| | 2 | 112.46 | 23.42 | 1.54 | 92.67 |
| | 3 | 116.38 | 2.06 | 1.81 | 97.34 |
| | 4 | 115.07 | 21.56 | 1.64 | 99.38 |
| MQC-11 (823.01 ng/ml) | 1 | 786.45 | 12.45 | 2.45 | 95.67 |
| | 2 | 798.56 | 44.56 | 2.04 | 96.43 |
| | 3 | 800.86 | 23.18 | 1.05 | 98.48 |
| | 4 | 798.27 | 67.43 | 0.83 | 98.04 |
| MQC-1 (7620.48 ng/ml) | 1 | 7552.23 | 46.23 | 1.28 | 91.06 |
| | 2 | 7597.56 | 55.46 | 0.81 | 92.07 |
| | 3 | 7601.45 | 12.45 | 1.59 | 97.56 |
| | 4 | 7592.64 | 45.57 | 2.45 | 93.45 |
| HQC (13 608.52 ng/ml) | 1 | 13 610.01 | 41.227 | 0.34 | 96.93 |
| | 2 | 13 556.00 | 294.06 | 2.16 | 91.06 |
| | 3 | 13 587.63 | 225.95 | 1.66 | 97.28 |
| | 4 | 1358.67 | 124.02 | 1.86 | 97.05 |
| | | | Within batch interday | | |
| LLOQ | | 37.49 | 7.45 | 2.08 | 97.21 |
| LQC | | 115.46 | 4.38 | 1.58 | 98.40 |
| MQC-1 | | 7609.01 | 13.12 | 5.57 | 94.20 |
| MQC-11 | | 796.53 | 11.23 | 2.46 | 95.76 |
| HQC | | 13 502.42 | 0.43610 | 9.51 | 91.66 |

%CV, percentage coefficient variation; HQC, high quality control; LLOQ, lower limit of quantitation; LQC, lower quantitation limit; MQC, medium quality control. [a]Each run includes six replicates.

**Table 3 Linearity data of the alogliptin**

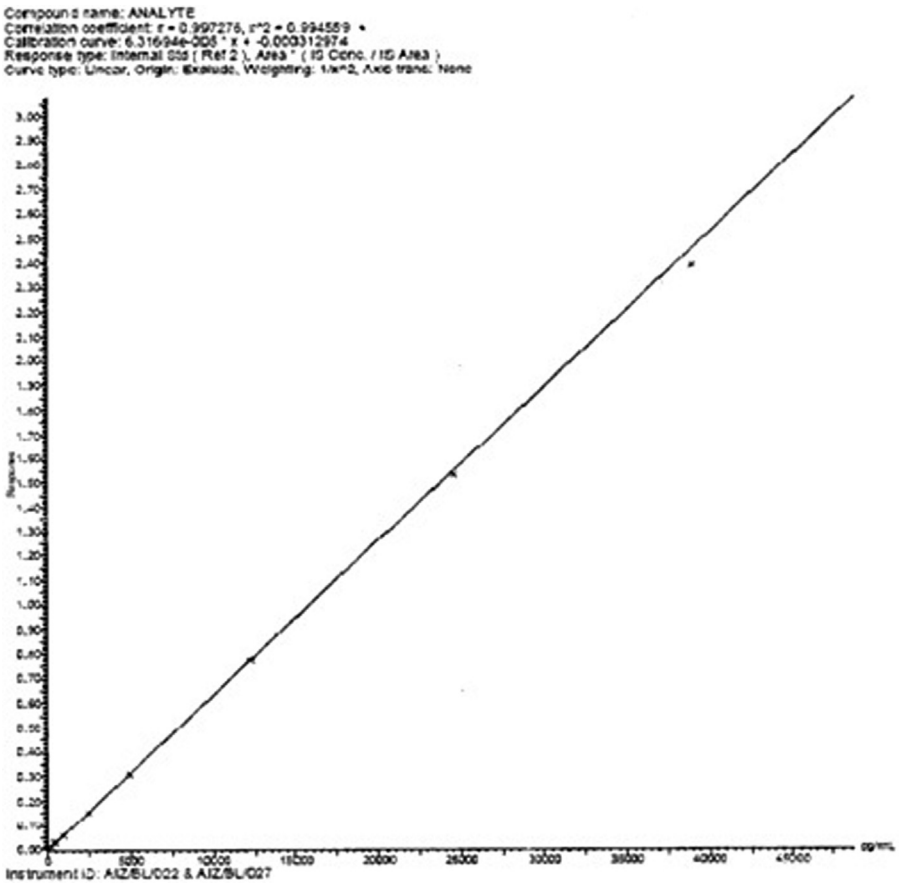
| Acquisition batch ID | STD1 | STD2 | STD3 | STD4 | STD5 | STD6 | STD7 | STD8 | STD9 | STD10 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Nominal concentration (ng/ml) | | | | | |
| | 40.176 | 80.352 | 160.705 | 321.409 | 803.524 | 1607.047 | 4017.618 | 8035.236 | 12 876.980 | 16 096.225 |
| | | | | | Nominal concentration range (ng/ml) | | | | | |
| | 32.141–48.211 | 68.299–92.405 | 136.599–184.811 | 273.198–369.620 | 682.995–924.053 | 1365.990–1848.104 | 3414.975–4620.261 | 6829.951–9240.521 | 10 945.433–14 808.527 | 13 681.791–18 510.659 |
| | | | | | Back-calculated concentration(ng/ml) | | | | | |
| P&A01REC01 | 38.1987 | 82.4013 | 166.280 | 336.064 | 814.262 | 1471.593 | 4082.257 | 8036.410 | 12 610.327 | 15 690.339 |
| P&A02ME01 | 39.450 | 80.967 | 168.413 | 326.890 | 806.085 | 1628.786 | 3966.119 | 7746.707 | 12 556.831 | 16 132.782 |
| P&A03SENO 1DI01 | 39.829 | 80.535 | 160.611 | 339.710 | 803.927 | 1631.156 | 4241.082 | 7664.337 | 12 690.309 | 15 122.952 |
| *N* | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Mean | 39.1987 | 82.4013 | 165.1013 | 334.2213 | 808.0913 | 1577.1783 | 4096.4860 | 7815.8180 | 12 619.1557 | 15 648.6910 |
| SD | 0.78671 | 2.86661 | 4.03234 | 6.60565 | 5.45180 | 91.44726 | 138.0365 | 195.42729 | 67.17554 | 506.20161 |
| %CV | 2.01 | 3.48 | 2.44 | 1.98 | 0.67 | 5.80 | 3.37 | 2.50 | 0.53 | 3.23 |
| %Mean Accuracy | 97.57 | 102.55 | 102.74 | 103.99 | 100.57 | 98.14 | 101.96 | 97.27 | 98.00 | 97.22 |

%CV, percentage coefficient variation.

### Stability studies

The % nominal for bench top stability study results was within 99.43 and 96.81% at HQC and LQC level. In the autosampler stability study, the calculated % nominal was found 97.03 and 93.92 for HQC and LQC samples. The results of four cycles freeze thaw stability sample exhibited an excellent range of acceptability in both LQC and HQC level of Alogliptin, with an accuracy value of 99.29 and 102.04%. In wet extract stability study results, % nominal value for alogliptin was more than 97%. The short-term stability, the calculated % nominal value was 98.23 and 96.32% at HQC and LQC level, respectively. The mean % nominal value for long-term stability study (30 days at −70°C) was found to be 93.59 for LQC and 95.28% for HQC

**Figure 5**




Linearity of alogliptin.

**Table 4 Stability data of alogliptin**

| | | Alogliptin | | | |
|---|---|---|---|---|---|
| QC levels | Type of stability | Mean[a] | SD | %CV | %Nominal |
| LQC (117.67 ng/ml) | Bench top | 112.45 | 0.71 | 1.93 | 94.53 |
| | Freeze thaw | 114.27 | 0.41 | 2.88 | 97.06 |
| | Autosampler | 112.08 | 0.61 | 2.62 | 94.80 |
| | Short term | 113.67 | 0.33 | 2.68 | 95.20 |
| | Long term | 113.48 | 0.53 | 4.04 | 92.52 |
| HQC (13 608.52 ng/ml) | Bench top | 13 575.06 | 0.32 | 2.58 | 98.56 |
| | Freeze thaw | 13 406.43 | 0.46 | 1.79 | 95.56 |
| | Autosampler | 13 397.21 | 0.36 | 4.80 | 93.91 |
| | Short term | 13 543.55 | 0.35 | 2.30 | 97.12 |
| | Long term | 13 600.61 | 0.22 | 1.55 | 99.16 |

%CV, percentage coefficient variation; HQC, high quality control; LQC, lower quantitation limit. [a]Mean of six replicates.

samples. The stability study results are included in Table 4.

## Discussion

After considering the results obtained the optimization of mass parameters and liquid chromatographic operating conditions was found suitable for the separation of analyte and IS with good peak shape, specific, and less retention time. All the studied validation parameters as per USFDA bi analytical method development guidelines were within the acceptance criteria. The matrix and analyte selectivity study results demonstrated that the developed method was found selective because there is a 0% of interference. The carryover test result shows 0% carryover of LLOQ and ULOQ samples of alogliptin and IS, which are found within the acceptance criteria. The result of IS normalized matrix factors value indicates that there was no such significant matrix ion effect for the alogliptin and IS. In the study of accuracy and precision,

between the batch and within batch precision and accuracy results in all quality control levels indicates that the % nominal and %CV values were within acceptance criteria and found highly precise and accurate. The results of mean overall recovery of alogliptin and ISs in LQC, MQC-1, MQC-2, and HQC level were found well satisfactory recovery and the % recovery differences were within acceptance criteria. The present investigated method was found linear in 40.176–16 096 ng/ml with regression coefficient 0.99 for alogliptin, which confirms the linearity and sensitivity of the developed method. In dilution integrity study, the quality control samples in all quality control levels maintained the integrity on dilution at 5 and 10 times because the % nominal and %CV values were within the acceptance criteria. In ruggedness study, the accuracy and precision results demonstrated satisfactory accuracy and precise values in all QC levels and found within the limits and claims the ruggedness of the method on changing different column and different analyst. In the stability study, the bench top, wet extract, autosampler, freeze thaw, and short-term and long-term stability studies, stability samples were compared with freshly prepared plasma samples, and concentrations were obtained through back calculation from the calibration curve samples. In all category of stability study, the mean of % nominal values and %CV values were within 10%, which strongly claimed about the stability of all quality control samples and as well as the stability of the developed method.

## Conclusion

Based on the factual evidences of all validation study results, the method can be consider validated because all the parameters are within the acceptance limit as per the USFDA guidelines. So, this present novel method can undoubtedly be highly applicable for the quantitative analysis of alogliptin and further applicable to quantify the alogliptin and for carrying out pharmacokinetic study in human and animal's plasma during clinical and preclinical trials, as well as forensic and toxicological study.

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### Conflicts of interest

There are no conflicts of interest.

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