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Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF VILDAGLIPTIN IN RAT PLASMA USING LCMS/MS METHOD

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ABSTRACT

A simple, wide range, selective, cost effective method using liquid chromatography tandem mass spectrometry method (LC-MS/MS), needs to be developed for toxicological studies in rat plasma due to unavailability of such kind of method in the industry. A sensitive, accurate and selective method was developed and validated for the quantitation of Vildagliptin (VLD) in rat plasma. Electrospray ionization (ESI) interface in positive ion mode was selected to improve selectivity and the sensitivity required for this application. Additionally, a protein precipitation was performed for sample clean up. The separation was achieved in less than 5 min using a ACE 3 C18 PFP, column and a mobile phase, composed of a mixture of ammonium acetate buffer: acetonitrile (20:80, v/v), in isocratic mode at a flow rate of 0.7 mL/min. Detection was carried out in mass spectrometer at m/z 304.4 (parent) and 154.1 (product) for Vildagliptin and 311.1 (parent) and 161.1 (product) for Vildagliptin D7. The retention time observed was 3.00 to 3.20 min. The standard curve was linear (R^2 >0.99) over the concentration range of 7.06 ng/mL to 3023.81 ng/mL. The method has shown tremendous reproducibility, with within- and between batch precision less than 9 %, and accuracy within ± 15% of nominal values and has proved to be highly reliable for the analysis of pre-clinical samples for toxicokinetic studies.

Keywords: Vildagliptin; LC-MS/MS; Method Development; Validation

1. INTRODUCTION

The purpose of this study was to develop an improved, sensitive and high throughput bio-analytical method for quantification of Vildagliptin in rat plasma by LC-MS/MS, in order to apply the methodology to assess the toxicological effects of Vildagliptin._Vildagliptin is an anti-hyperglycemic agent which belongs to new dipeptidyl peptidase-4 (DPP-4) inhibitor class of drugs. Vildagliptin is a molecule which manages the glucagon levels both in hypoglycemia and hyperglycemia [1]. Vildagliptin inhibits the inactivation of GLP-1 and GIP by DPP-4, increases the secretion of insulin by beta cells and glucagon release is suppressed in pancreas by the alpha cells of the islets of Langerhans. GLP-1 and GIP hormones are secreted in small intestine and when glucose level increases, they are responsible to increase the insulin level to control blood glucose level. Vildagliptin is used in type 2 diabetes mellitus [2].

Vildagliptin given orally is rapidly absorbed in the body. It has over 90% oral bioavailability in the body, but It shows very little tendency to bind with plasma protein which is around 9%. The elimination half-life of this drug is approximately 1.5–4.5 h [3]. Its soluble in water is around 1.75 mg/mL and it is also soluble in dimethyl sulphoxide [4]. A variety of analytical methods using high performance liquid chromatography coupled with ultraviolet (UV) detection are available. Most analytical methods were developed on pharmaceutical dosage form and not on the plasma [5-11]. Some of the bioanalytical methods were developed and reported on Vildagliptin [12-16]. The published methods with UV were proved to be time consuming and higher limit of quantification. The reported methods are time consuming, higher limit of quantification and utilizes electrospray ionization interface in positive ion mode towards the quantification of Vildagliptin [16]. These methods require higher plasma aliquot volume, tedious and time-consuming extraction procedures and/or are not sensitive enough or not as per expected range for the estimation of Vildagliptin.

In this research, our aim was to develop a sensitive and precise LCMS/MS methodology for determination of

Vildagliptin with an electrospray ionization (ESI) interface in positive ion mode in shorter run time which gives high throughput. The validated method was successfully applied to a pre-clinical toxicokinetic study in rat.

2. MATERIAL AND METHODS

2.1. Materials

Vildagliptin working standard was procured from Vivan Life Sciences Pvt. Ltd., Thane, Mumbai, India, whereas its deuterated standard for internal standard (ISTD, Vildagliptin-D7) was procured from Clearsynth Labs, Andheri, Mumbai, India. Fig. 1 (a) and (b) represents chemical structure of Vildagliptin and Vildagliptin D7.

Ammonium acetate and acetonitrile of LC-MS grade were obtained from Sigma-Aldrich, Formic Acid of AR grade was procured from Rankem. All aqueous solutions and buffers were prepared using water that was purified using Milli-Q Gradient A10 (Millipore, Molscheim, France). RAT plasma matrix lots for method development, validation, calibration standards and quality control (QC) samples were obtained from Drug and Safety evaluation department of Sun Pharmaceutical Industries Limited, Gurugram, Haryana, India.

2.2. LC-MS/MS instrumentation and operating conditions

The liquid chromatography separation was performed using Applied Biosystems LC-MS/MS system (Shimadzu Corporation; Kyoto, Japan) comprising of two LC-20AD pumps, a cooling autosampler (SIL-20AC), a column oven of temperature control (CTO-20AC) and a CBM-20 A controller. Chromatography separation of analytes and their corresponding d7-ISTDs was accomplished within 4.50 min using an ACE 3 C18 PFP (150 mm × 4.6 mm, 3µm; Advanced Chromatography Technologies Ltd, UK) column and a mobile phase consisting of mixture of ammonium acetate buffer: acetonitrile (20:80, v/v), in isocratic mode at a flow rate of 0.7 mL/min. The column and autosampler temperature were kept at 40°C and 10°C, respectively.

Mass detector of model API 4000 (MDS-Sciex®, Concord, Canada) consisting of an ESI interface was operated in positive ion mode. Quantification was carried out using multiple reaction monitoring (MRM) mode of the transition's m/z 304.4/154.1 for Vildagliptin, m/z 311.1/161.1 for Vildagliptin-D7 with dwell time set at 400 ms per transition. Nitrogen was used as the nebulizer, auxiliary, collision and curtain gases. The main working source/gas parameters of the mass spectrometer were optimized and maintained as follows: collision activated dissociation (CAD) gas, curtain gas (CUR), gas 1 (nebulizer gas), gas 2 (heater gas), turbo ion spray (IS) voltage, 4000 V; and source temperature, 500°C. Other optimized compound parameters for monitoring analyte were set as follows: declustering potential (DP), 70 V; entrance potential (EP), 6 V; collision energy (CE), 24 V; and collision cell exit potential (CXP), 12 V.

Calibration curves were constructed by calculating the analyte to internal standard (ISTD) peak area ratio (y) against analyte concentrations (x). Data acquisition and processing were performed using Analyst version 1.4.1 software (MDS-Sciex®, Canada).

2.3. Preparation of stock and working solutions, standard and quality control samples

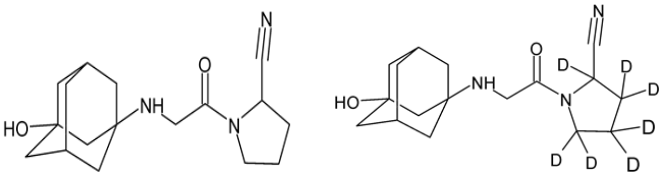
Stock solutions of Vildagliptin and their ISTDs (Vildagliptin-D7) were prepared separately by dissolving the accurately weighing the compounds in methanol to obtain a final concentration of 1 mg/mL. The prepared stock solutions were stored at 2-8°C until use. Working solutions were then prepared in acetonitrile-water (20:80, v/v) for the preparation of calibration standards. One set contained nine concentration levels for Vildagliptin. Calibration standards containing analytes were prepared by 5% addition of each working solution in rat blank plasma (e.g., at each concentration level, 50µL of each of the working solutions was added to 1mL rat plasma). This resulted in the calibration range 7.06-3023.81 ng/mL. Simultaneously quality control (QC) samples were prepared in the same manner as that of calibration standards in bulk from the working solutions at five levels viz. High/HQC (2513.47 ng/mL), Middle/MQC (1005.39 ng/ml), Low/LQC (19.71 ng/mL) and limit of quantitation quality control LOQQC" (7.09 ng/mL).

The calibration standards and quality control samples were stored in aliquots at -50°C until analysis. The stock solutions used for the preparation of quality control samples were different from the one used for the preparation of calibration curve standards.

A working solution containing the ISTDs was prepared in acetonitrile: water (20:80, v/v) at concentrations of 250.00 ng/mL for Vildagliptin -D7.

2.4. Protein precipitation procedure

Plasma samples frozen at -50°C were thawed on the day of extraction at room temperature. The thawed samples were vortexed to ensure complete mixing of contents.



(a) (b)

Fig. 1: Chemical structure (a) Vildagliptin and (b) Vildagliptin D7

Eppendorf pipette was used to pipette out 20 µL aliquot of each plasma sample into appropriately labelled polypropylene tubes. 50 µL of internal standard dilution containing ~250.00 ng/mL of Vildagliptin-D7 (except in double blank in which 50µL dilution solution was added) was added. 1.0 mL of acetonitrile was added, and samples were vortexed to ensure complete mixing of contents. The samples were centrifuged at 20000 rcf for minimum of 5 min. 200 µL of supernatant was pipetted out into appropriately labelled polypropylene tubes and dried at 50°C and 15 psi using a Zymark TurboVap LV evaporator (Caliper, Hopkinton, MA, USA). The dried samples were reconstituted with 500µL of the mobile phase and samples were transferred into polypropylene vials for analysis.

2.5. Effect of storage on bench top for different time and multiple freeze-thaw cycles

Freeze thaw stability was performed for at least three cycles at low and high QC level. The samples were frozen and thawed over three freeze and thaw cycles and then samples were kept for bench top stability for certain period at room temperature. After this, samples were analysed with freshly spiked and the changing trends of analyte peak area response were plotted against respective variables.

2.6. Method validation

A full method validation was performed according to guidelines set by the USFDA. The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, and stability of analyte during both short-term sample processing and long-term storage.

2.6.1. Selectivity

The selectivity of the assay was evaluated by analysing blank plasma samples (six normal, one lipemic and one haemolyzed) from eight different rats and spiked plasma samples at lower limit of quantitation (LLOQ) level. The peak area of the co-eluting components should be less than 20% and 5% those of the analytes and the corresponding ISTD, respectively.

2.6.2. Linearity and sensitivity

The linearity of the method was determined by analysis of standard plots associated with eight-point calibration curve. Calibration curves from accepted three precision and accuracy batches were used to establish linearity. Peak area ratios of analyte/ISTD obtained from MRM were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations and the measured peak area ratios. Back-calculations were made from these curves to determine the concentration of analytes in each calibration standards and the resulting calculated parameters were used to determine concentrations of analyte in quality control or unknown samples. The correlation coefficient (r)> 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was to be accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with accuracy within ± 20% and a precision ≤ 20%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15%.

2.6.3. Accuracy and precision

Intra- and inter-day accuracies were expressed as a percentage of deviation from the respective nominal value and the precision of the assay was measured by the percent coefficient of variation (% CV) at concentrations. Intra-day precision and accuracy was assessed by analysing six replicates of the quality control samples at four levels during a single analytical run. The inter-day precision and accuracy was assessed by analysing 18 replicates of the quality control samples at each level through three precision and accuracy batches runs on 2 consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ± 15 % except LOQQC, for which it should not be more than 20 %. Similarly, the mean accuracy should not deviate by ± 15 % except for the LOQQC where it can be ± 20 % of the nominal concentration.

2.6.4. Recovery

Recovery was estimated at three QC concentration levels (low, medium, and high) by comparing the mean

peak area of all the analytes in the QC samples (n = 6) with post extracted spiked blank samples representing 100 % extraction of analytes at each QC concentrations. The recoveries of D7-ISTD were measured in a similar manner using their corresponding medium QC samples as reference.

2.6.5. Matrix effect and matrix factor

Matrix effect was assayed at two concentration levels (LOQQC and HQC). Six different plasma lots (4 normal, 1 haemolyzed, 1 lipemic) free of any significant interference at the room temperature of analytes and ISTDs, were selected and spiked with the working solutions of LOQQC and HQC. Spiked samples from each plasma lot were processed in duplicate and quantitated against freshly spiked calibration curve. The matrix effect was validated to be nullified if the accuracy and precision does not deviate by ± 15 % for HQC and ± 20 % for LOQQC of the nominal concentration. The matrix factor was defined as the peak area response in the presence of matrix ions versus the peak area response in the absence of matrix ions. Since this method involved terminal drying step, biological matrix samples were prepared by reconstituting the post-extracted blank plasma samples with neat solutions (n = 3) containing analytes and ISTDs at equivalent concentration representing the 100% recovery in final extracted concentration for the analytes (low, medium, and high QC level) and ISTDs. The control samples were the same neat solutions prepared in reconstitution solution. Matrix factor was evaluated using six different blank plasma lots (4 normal, 1 haemolyzed, 1 lipemic) and determined by measuring the respective mean peak area response (absolute matrix factor) and mean analyte/ISTD peak area ratio (ISTD normalized matrix factor) of biological matrix sample against the mean peak area response and mean analyte/ISTD peak area ratio of neat solutions.

2.6.6. Stability

Stability of analytes was evaluated using QC samples. Bench-top stability was evaluated at room temperature for more than 6 h, which exceeds the residence time of the sample processing procedures. The freeze-thaw stability was evaluated after undergoing three freeze (at -50°C)-thaw cycles. Long-term stability was assessed after storage of the test samples at below -15°C and -50°C for 36 days. The autosampler storage stability was determined by storing the reconstituted QC samples for ~74.58 h under autosampler condition (maintained at 10°C) before being analysed. All stability exercises were performed against freshly spiked calibration standards processed along with freshly spiked comparison QC samples at low and high concentrations (n = 4) for determining the absolute stability of analytes. Absolute stability was calculated as follows:

% absolute stability = (average concentration of stability samples/average concentration of comparison samples × C.F.) × 100;

C.F. = concentration of stability sample/concentration of comparison sample.

The analyte was considered stable at each concentration if the mean calculated concentration of stability samples was not deviated by ±15% of the mean calculated concentration of comparison quality control samples. The working solutions and stock solutions of analytes and ISTDs were also evaluated for their stability at room temperature for about 3.05 h and at 2-8°C for 48 days, respectively.

3. RESULTS AND DISCUSSION

3.1. Method development

3.1.1. Mass spectrometry optimization

During the early stage of method development, ESI offered much higher signal intensities for all target analytes and was thus chosen as the ionization source. The first LC-MS/MS tests to select the optimum MS/MS parameters and the appropriate ions were carried out by syringe pump infusion of standard solution at a concentration of 100 ng/mL in mobile phase, with monitoring of MS intensity. It was found that the best results in terms of peak area and peak height were obtained in ESI positive ion mode than in ESI negative ion mode due to their readiness to accept proton. In this context, ESI positive ion mode was chosen. Figure 2 (a) and (b) shows the product ion spectra of the analytes and their deuterated ISTDs, Deuterated ISTDs shared similar fragmentation patterns.

For MRM detection, the most stable and appropriate fragment ions produced by the analytes and ISTDs were selected. Different additives of varying strength were added to the mobile phase, to obtain higher abundance of protonated parent ion of analytes and ISTDs. Use of additives like acetic acid, ammonium formate, ammonium acetate, etc., in mobile phase enhances the occurrence of $[M-H]^+$ and eventually results in an optimal area response for analytes and ISTDs.

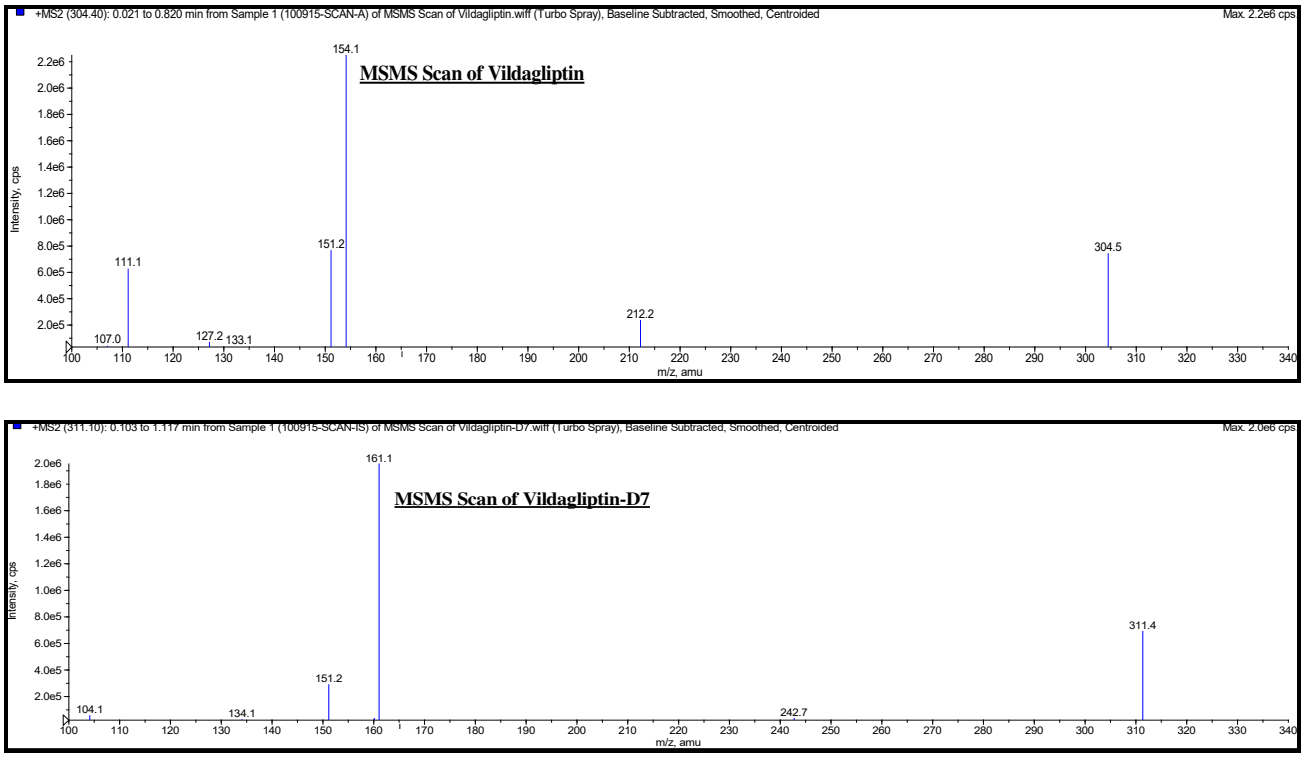


Fig. 2: Product ion spectra (a): Vildagliptin, (b): Vildagliptin-D7

3.1.2. Liquid chromatography conditions

Several reversed-phase columns were tested to achieve optimal resolution, selectivity, and efficiency with a short running time. ACE 3 C18 PFP was chosen as an analytical column because it offers a high separation power with modest operating pressure and involves the usual compromise of chromatographic characteristics.

The optimization procedure was focused on the mobile phase composition, column oven temperature, and injection volume. Firstly, several mobile phases composed of acidified water with appropriate organic phase (acetonitrile/methanol) in different ratios were tested. After several tests had been performed, it was possible to obtain the desired resolution and sensitivity with mobile phase composed of a mixture of ammonium acetate buffer: acetonitrile (20:80, v/v), in isocratic mode at a flow rate of 0.7 mL/min. Simultaneously, tests were carried out to study the influence of the column temperature (between 30 and 50°C, at an increment of 5°C) on retention times and injection volume (from 5 to 20 µL) towards enhancing the sensitivity of the method. The best results were achieved when 5 µL was injected and column oven maintained at 40°C. Under the chromatographic conditions described above, the D7-ISTDs were eluted at the same retention time as their corresponding unlabeled analytes.

The retention times were 3.0 to 3.2 min for both analyte and ISTD (Figure 3).

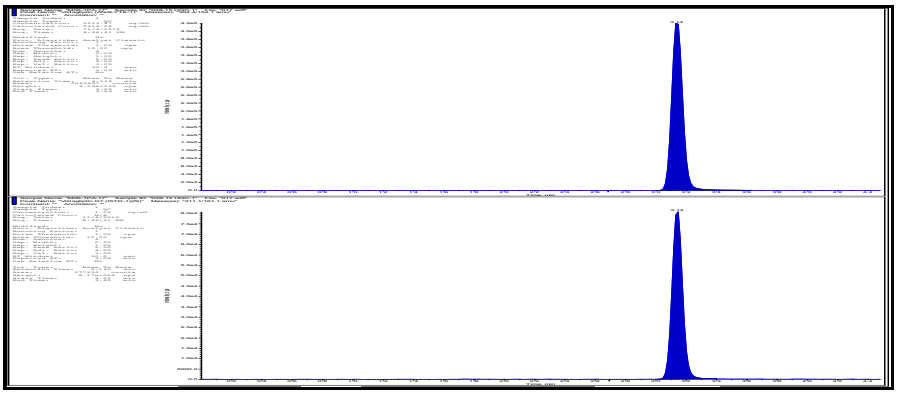


Fig. 3: Representative chromatograms of HQC

3.1.3. Sample preparation

During method development different options were evaluated to optimize sample cleanup to eliminate possible matrix interferences, concentrate the sample and obtain cost effective method. Firstly, the simplest and fastest protein precipitation (PPT) method for preparing samples was carried out which worked well hence selected the method for sample preparation [17-18].

3.2. Method validation

3.2.1. Selectivity and sensitivity

Blank plasma including one lipemic and one hemolyzed did not show interference at the retention times of Vildagliptin and Vildagliptin-D7 (ISTD), no interference at the analyte retention time by internal standard was observed. Refer figure 4 (a) and (b) for blank and blank with internal standard sample.Ratio of S/N of each LOQ sample and mean S/N ratio of normal blank matrix samples (taken from selectivity and S/N ratio exercise) was calculated; and was > 5 for all LLOQ samples. The limit of quantitation was 7.06 ng/mL for Vildagliptin, refer figure 5 for LLOQ chromatogram.

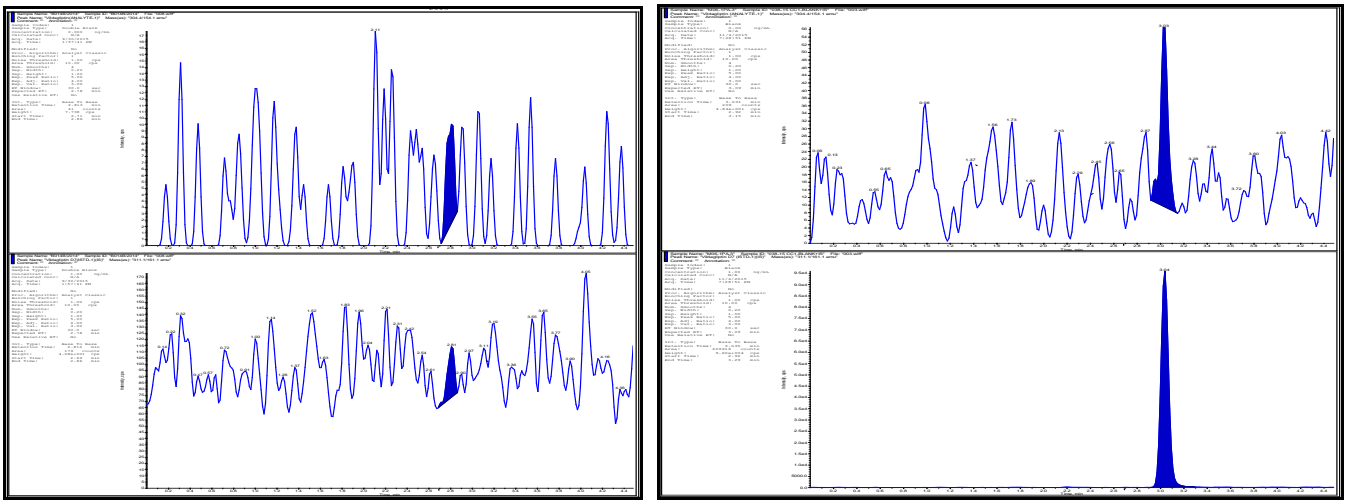


Fig. 4: Representative chromatogram of (a) blank (b) blank with internal standard

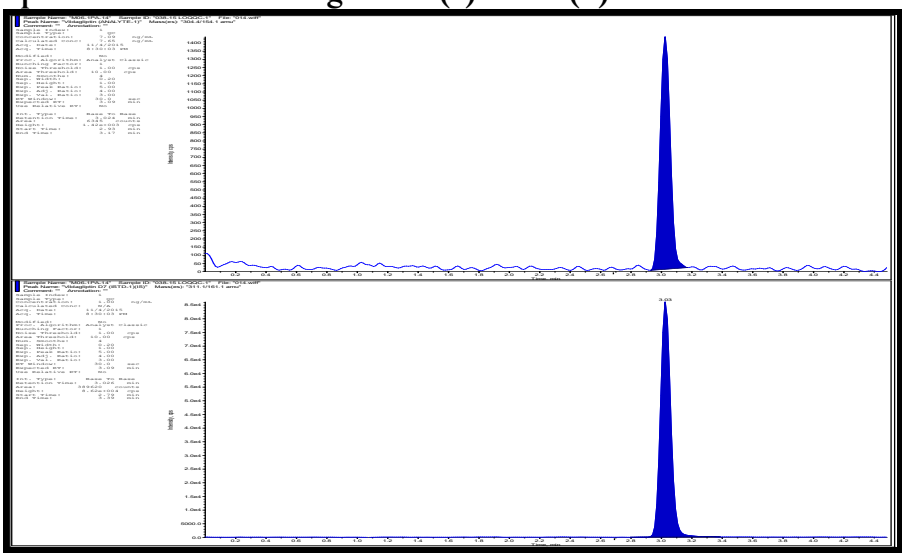


Fig. 5: Representative chromatogram of LLOQ

3.2.2. Linearity

The linearity of the method was determined by a weighted least square regression analysis of standards curve consisting of eight concentrations.Three calibration curves of accepted precision and accuracy batches were used to establish linearity. Linear regression algorithm was selected. The weighting factor was evaluated for Vildagliptin. A weighting factor of $1/x^2$ was selected. The method was found linear throughout the range. Refer fig. 6 for calibration curve.

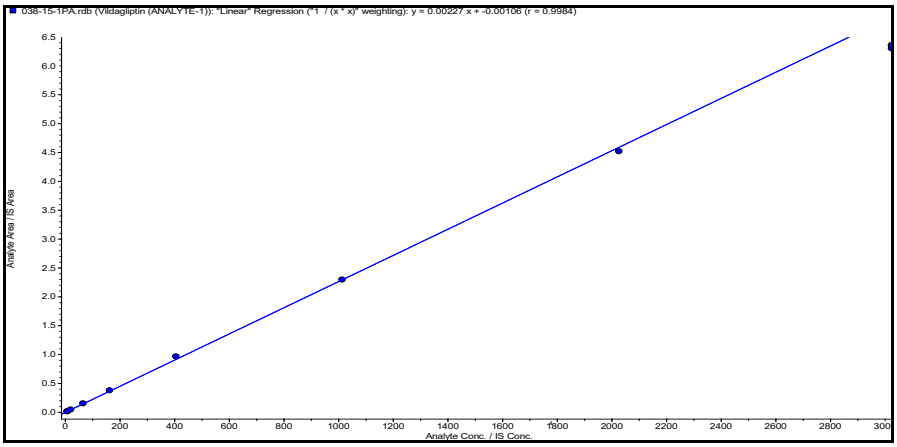


Fig. 6: Linearity curve of Vildagliptin

3.2.3. Precision and Accuracy

Eighteen replicates of the QC samples from three consecutive validation runs were used to evaluate precision and accuracy at each concentration level. Intra-day precision and accuracy of Vildagliptin ranged from 1.36 % to 4.62 % and 85.54 % to 105.15 %, respectively. Inter-day precision and accuracy of Vildagliptin ranged from 2.81 % to 7.15% and 91.10 % to 103.90 %, respectively. Intra-day and inter-day precision and accuracy data is presented in table 1.

Table 1: Intra- and inter-day precision and accuracy data for the determination of Vildagliptin

Compound	Spiked Concentration (ng/mL)	Intra-day (*n*=6)			Inter-day (*n*=18)		
		Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)
Vildagliptin	7.09	7.455	105.15	4.62	7.367	103.90	7.15
	19.71	19.925	101.09	3.54	20.287	102.93	5.37
	1005.39	1038.758	103.32	1.67	1043.827	103.82	3.92
	2513.47	2381.890	94.77	3.06	2483.199	98.80	3.71
	5032.48	4304.767	85.54	1.36	4584.372	91.10	5.75
	5032.48	4589.742	91.20	2.64	4695.697	93.31	2.81

3.2.4. Matrix effect and matrix factor

Matrix effect as well as matrix factor (MF) on the presented method was evaluated following the procedures described above. Results obtained therein indicated that no additional variations in plasma concentration due to the use of different plasma lots were observed. Precision and Accuracy for matrix effect samples at LOQQC level 7.45% and 94.61% and at HQC level 5.83% and 90.84% for Vildagliptin, were found within the acceptable range. Hence the concentration of the analyte obtained was reliable. MF of analyte(s) and ISTD(s) was calculated individually and 'ISTD normalized MF' (ISTD-MF) at low, middle and high QC concentration level for each blank matrix lot was calculated. Mean, S.D., N and % C.V. of ISTD-MF were calculated for all blank matrices. The % C.V. of ISTD-MF was 2.32 and mean of ISTD-MF was 1.000 for Vildagliptin.

3.2.5. Recovery

The results of recovery exercise for Vildagliptin at LQC, MQC and HQC level were 97.20 %, 103.50 % and 109.64 %, respectively. Mean and % C.V. of % recovery across the low, middle and high-quality control concentration were found to be 103.44 and 6.01% for Vildagliptin, respectively.

3.2.6. Stability

The results of stability tests evaluated and pointed out that, all analytes spiked into rat plasma were stable for at least 6.28 h at room temperature, in an autosampler post extraction for 74.58 h at 10°C. Stability of analyte in plasma at below -50°C and -15°C was found up to 36 days and up to three freeze-thaw cycles (-50°C). The stock solutions of all analytes and ISTDs were found stable at 2-8°C for 48 days and the working solutions of all analytes and ISTDs were found stable for about 3.05 h at room temperature.

4. CONCLUSION

Vildagliptin is an antidiabetic drug which is used in type II diabetes. A bio-analytical method was developed and validated for the estimation of Vildagliptin in rat plasma using LC-MS/MS. The method was developed using mobile phase composition Acetonitrile: 10 mM Ammonium acetate buffer (80:20 v/v). The peaks obtained for the drug of interest and internal standard were not having any interference at each other transition. Protein precipitation technique was used for extraction of analyte from plasma sample. The peak shape was good and symmetrical in nature. All the analytical validation parameters were performed and found within acceptance limit. The method was validated with respect to selectivity, linearity, accuracy, precision, and stability. The results were within the limits. The method was found linear with a correlation coefficient close to 1 in the given range. Thus, method is suitable for the estimation of Vildagliptin in rat plasma. The lower limit of quantification (LLOQ) of Vildagliptin was achieved and found within acceptance limit. The method was selective, specific for the estimation of Vildagliptin in rat plasma and other biological fluids. From the current work, it was concluded that the developed LCMS/MS method in rat plasma using protein precipitation for sample preparation was found to be very simple, reliable, precise, accurate, sensitive and selective for the estimation of Vildagliptin. The method is suitable for routine quantitative analysis. The method developed can be used in bioavailability and toxicological studies in rat.

5. ACKNOWLEDGEMENT

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