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HIGH THROUGHPUT LCMS-MS METHOD FOR THE QUANTITATION OF SAXAGLIPTIN IN RAT PLASMA BY PROTEIN PRECIPITATION USING 96 WELL PLATE FORMAT

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ABSTRACT
High-throughput Liquid chromatography–mass spectrometry method has been developed and validated for the quantification of Saxagliptin in rat plasma using Sitagliptin as internal standard (ISTD). Following protein precipitation in 96 well plate format, the analytes and ISTD were run on ACE C18 4.6 x75 mm (5.0 µm) using an isocratic mobile phase consisting of 2 mM Ammonium Formate with 0.1% Formic Acid and Acetonitrile (50:50 v/v). The precursor and product ions of the drugs were monitored on a triple quadrupole instrument operated in the positive ionization mode. The method was validated over a concentration range of 0.997 to 249.259 ng/mL with mean recovery of 104.74%. The inter batch precision (%CV) across three validation runs was ≤ 10.5%. The Inter batch accuracy determined at four QC levels (LLOQ, LQC, MQC and HQC) was between 101.3 – 103.6%. According to the validated results, the proposed method was found to be specific, accurate, precise and high throughput method. This method could be used for the estimation of Saxagliptin in rat plasma and can be applied for the routine analysis.

KEYWORDS: Saxagliptin, Sitagliptin, Protein Precipitation and 96 well plates.

1. INTRODUCTION
Saxagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor antidiabetic for the treatment of type 2 diabetes. DPP-4 inhibitors are a class of compounds that work by affecting the action of natural hormones in the body called incretins. Incretins decrease blood sugar by increasing consumption of sugar by the body, mainly through increasing insulin production in the pancreas, and by reducing production of sugar by the liver. [Bristol-Myers Squibb Press Release] DPP-4 is a membrane associated peptidase which is found in many tissues, lymphocytes and plasma. DPP-4 has two main mechanisms of action, an enzymatic function and another mechanism where DPP-4 binds adenosine deaminase, which conveys intracellular signals via dimerization when activated. Saxagliptin forms a reversible, histidine-assisted covalent bond between its nitrile group and the S630 hydroxyl oxygen on DPP-4. The inhibition of DPP-4 increases levels active of glucagon like peptide 1 (GLP-1), which inhibits glucagon production from pancreatic alpha cells and increases production of insulin from pancreatic beta cells.[1, 2]

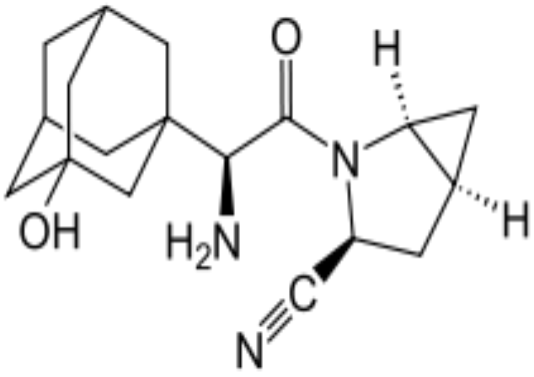

Figure 1: Structure of Saxagliptin

Very few methods have been developed for the estimation of saxagliptin in plasma by LCMM-MS.[3] The aim of the present work was to develop and validate the high throughput LC-MS/MS Method using 96 well plate protein precipitation plates as per the US FDA[4]

guidelines to quantify the Saxagliptin in rat plasma using sitagliptin as an internal standard.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Working standards of Saxagliptin and Sitagliptin were obtained as a gift sample from Hetero drugs (Hyderabad, India). Strata Impact Protein Precipitation plates were obtained from Phenomenex. LC–MS grade acetonitrile was purchased from Thermo Fisher Scientific India Pvt. Ltd.

(Mumbai, India). GR grade ammonium formate was pro-cured from Merck Specialties Pvt. Ltd. (Mumbai, India). Formic acid was obtained from Sigma Aldrich. HPLC water was obtained from Milli-Q water purification system (Millipore). Rat plasma containing K2 EDTA anticoagulant was obtained from Aptus Biosciences (Hyderabad, India).

2.2 Instrumentation

Agilent 1200 Series equipped with a binary pump for solvent delivery was used for the analysis. Mass spectrometric detection was performed on API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray inter-face. Quantitation was performed in multiple reaction monitoring (MRM) mode and Analyst software version 1.5.1(SCIEX) was used for controlling the hardware and data handling.

2.3 Chromatographic conditions

Chromatographic separation was performed on ACE C18 4.6 x 75 analytical columns. Isocratic mobile phase consisting of 2mM Ammonium formate with 0.1% formic acid and Acetonitrile was delivered at a flow rate of 0.5 mL/min. The auto sampler was set at $4^0C \pm 2^0C$ and the injection volume was 5 µL. The column oven temperature was set at 30.0 ± 2.0°C.

Retention Time of Saxagliptin was 1.38 and Sitagliptin was 1.56. The total chromatographic run time was 3.0 min.

2.4 Mass spectrometric conditions

2.4.1 Ionization mode: Positive ionization

2.4.2 Resolution: Q1 Unit; Q3 Unit

2.4.3 MRM conditions

Parameters	Q1 (amu)	Q3 (amu)	Dwell Time (msec)	DP (volts)	CE (volts)	CXP (volts)	EP (volts)
Saxagliptin	316.160	180.0	200	71	31	14	10
Sitagliptin	408.142	235.0	200	31	27	22	10

2.4.4 Source/ Gas parameters

Parameters	CUR (psi)	GS1 (psi)	GS2 (psi)	IS (Volts)	CAD (psi)	TEMP (°C)
Source/Gas	20	45	55	5500	6	400

2.5 Preparation of calibration standards and quality control samples

Standard stock solutions of Saxagliptin and internal standard (Sitagliptin) were prepared by dissolving their accurately weighed amounts in methanol to give a final concentration of 1mg/mL. Individual working solutions of analyte were prepared by appropriate dilution of their stock solutions in 50% acetonitrile. All the solutions were stored in refrigerator at below 10◦ C and were brought to room temperature before use. Working solution of internal standard (Linagliptin, 25 ng/mL) was prepared daily in 50% acetonitrile and was stored at room temperature.

Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with the working solutions (5%) prepared from independent stock weightings. K2 EDTA anticoagulant blank plasma was collected from rat. Calibration standards were prepared in plasma at concentrations of 0.997, 1.994, 4.985, 9.970, 19.941, 49.852, 99.704, 199.407 and 249.259 ng/mL. Quality control samples were prepared at 0.997 ng/mL (LLOQ QC), 2.792 ng/mL (LQC), 109.674 ng/mL (MQC) and 189.437 ng/mL (HQC).

2.6 Sample Preparation.

2.6.1 Label the 96 Well plates for sample processing.

2.6.2 Thaw and mix control matrix (K2 EDTA Rat Plasma) at room temperature.

2.6.3 Centrifuge the samples to remove particulates if necessary.

2.6.4 Prepare CC and QC samples by spiking 5 µL of working solution to 95 µL of control matrix.
2.6.5 Add 100 µl of 0.1% FA in Acetonitrile in Phenomenex Strata Impact PPT plate.
2.6.7 Add 20 µL of internal standard (25 ng/mL Sitagliptin) to all wells into which samples will be added except blank samples.
2.6.8 Transfer 100 µL of control matrix for blank and zero standards, 25µL of Spiked CC, QC and Study Samples plasma into the PPT plate containing mixture of internal standard and 0.1 %FA in Acetonitrile.
2.6.9 Wait for 1 minute.
2.6.10 Apply positive pressure using Ezypress HT 192 and elute the sample into collection plate.
2.6.11 Load the collection plate into Autosampler. Inject 5 µL of the sample onto the LC- MS/MS system.

2.6 Method validation

A complete method validation of Saxagliptin in rat plasma was done following the USFDA and EMEA guidelines. Validation runs were performed on seven separate days to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity and stability. Each validation run was organized with a set of spiked standard samples, blank (with ISTD and without ISTD) and QC samples as per the validation parameter. Standard samples were analyzed at the beginning of the run and QC samples were distributed consistently throughout the validation runs.

Selectivity of the method toward endogenous and exogenous components of plasma was evaluated in 6 different plasma lots. The blank plasma lots were extracted (without addition of ISTD), and injected for LC–MS/MS detection. Later selectivity in each lot was evaluated by comparing the blank peak responses against the mean peak response observed in plasma spiked LLOQ sample (n = 6).

Linearity of the method was assessed using three calibration curves analyzed on three different days. Each plot was associated with a nine point non-zero concentrations spread over the dynamic range. A quadratic regression analysis with reciprocate of drug concentration as weighing factor ($1/X^2$) was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentration of analytes over the established range.

Intra batch (within day) and inter batch (between day) precision and accuracy was evaluated at four distinct concentrations (LLOQ, LQC, MQC, HQC). Precision and accuracy at each concentration level was evaluated in terms of %CV and relative error respectively. The extraction recovery of Saxagliptin was determined at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with that of un-extracted samples (blank extracts spiked after extraction).

The matrix effect was checked at low and high QC level using six different blank plasma lots (including one hemolytic and one lipemic lot). Matrix factor for analyte and internal standard was calculated in each lot by comparing the peak responses of post extraction samples (blank extracts spiked after extraction) against the peak responses of equivalent aqueous samples prepared in mobile phase. Internal standard normalized matrix factor in each lot was later evaluated by comparing the matrix factor of analyte and internal standard.

Stability of analytes in both aqueous solutions and in biological matrix was evaluated after subjecting to different conditions and temperatures that could encounter during regular analysis. Stability in plasma was evaluated in terms of freeze–thaw stability, bench top stability, long-term stability, and extracted sample stability. Freeze–thaw stability was evaluated after seven freeze (at -70^{o}C) thaw (at room temperature) cycles. Bench top stability was assessed at room temperature and the long-term stability was evaluated at both -70^{o} C and -20^{o} C. Stability of extracted samples was determined after reconstitution (in-injector stability at 4^{o}C). Stability in whole blood was evaluated at room temperature. All the stability assessments were made at LQC and HQC level by comparing the stability samples against freshly prepared samples.

Stability of analytes in stock solutions and in working solutions was assessed at room temperature (short-term stability) and at 2-8^{o}C (long-term stability). All comparisons were made against freshly prepared stock solutions or working solutions. Before each analytical run, system suitability was evaluated by injecting six replicates of MQC sample to check the system precision and chromatography. System suitability was

considered acceptable when the coefficient of variation for response ratios was less than 4.0%.

3 RESULTS AND DISCUSSION

3.1 Method development

For consistent and reliable estimation of analytes it was necessary to give equal importance for optimization of extraction procedure along with chromatographic and mass spectrometric conditions. Analyte and ISTD were tuned in positive polarity mode using electrospray ionization technique. The Q1 and the MSMS scans were made in infusion mode and further compound and gas parameters were optimized in flow injection analysis. The [M+H] peaks were observed at m/z of 316.160 and 408.142 for Saxagliptin and Sitagliptin respectively. Most abundant product ions were found at m/z of 180.0 and 235.0 for both Saxagliptin and Sitagliptin (Fig. 2 and 3) by applying sufficient collision activated dissociation gas and collision energy. Increase in source temperature beyond 450^{0}C augmented the intensity. A 5% change in ionspray voltage and gas parameters did not affect the signal intensity.

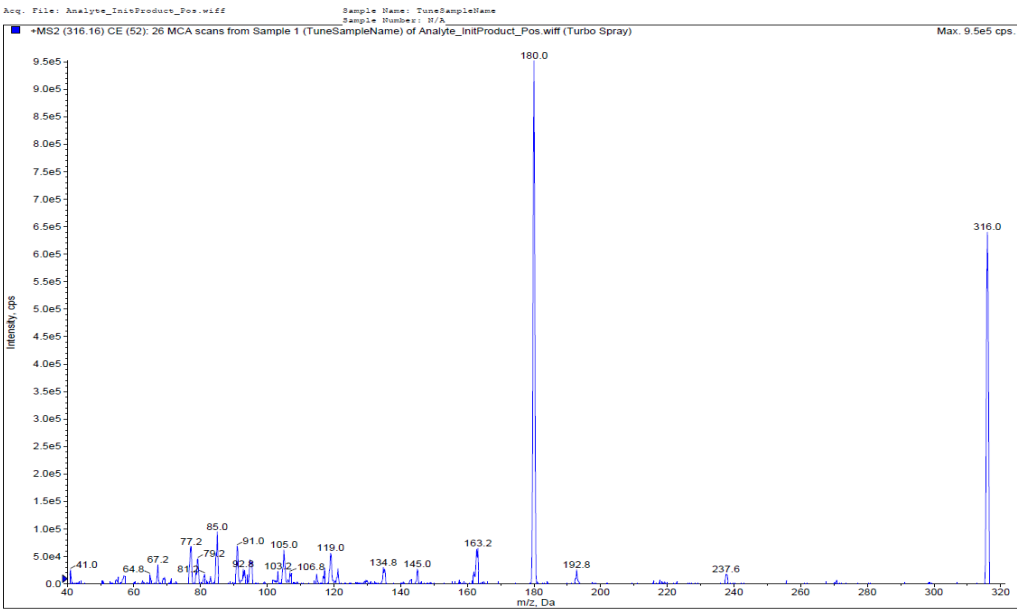

Fig 2: SAXAGLIPTINMS/MS SCAN

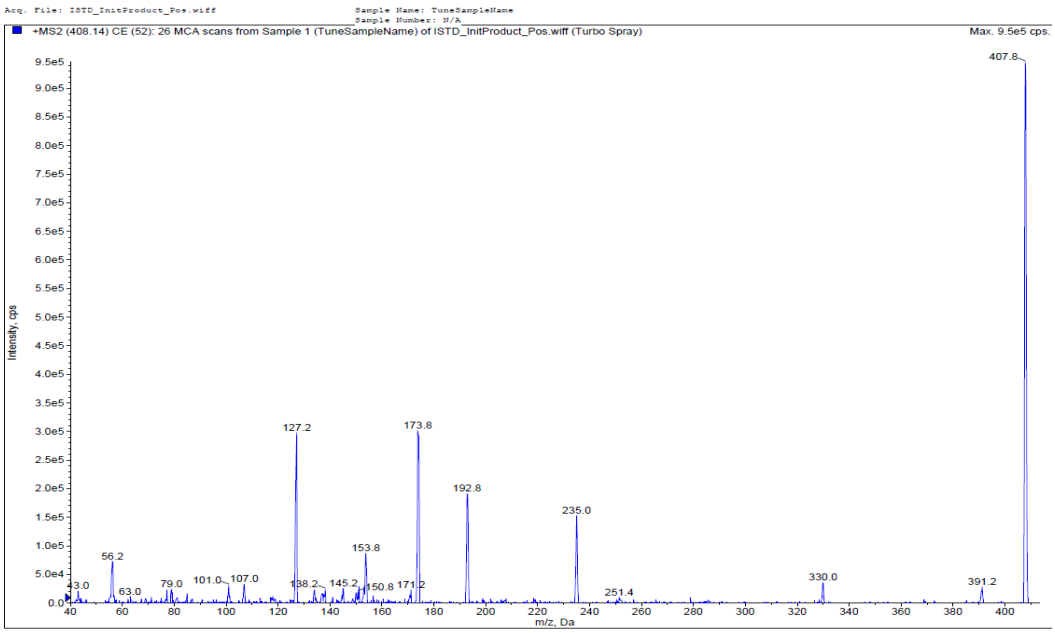

Fig 3: SITAGLIPTIN MS/MS SCAN

In the optimization of chromatographic conditions, isocratic mode was selected as no cross talk was observed between analytes and ISTD. Use of acetonitrile over methanol in the mobile phase has shown significant improvement in the signal intensities. Replacement of milli-Q water with 2 mM ammonium formate buffer in mobile phase gave good chromatographic peak shapes and further increase in the buffer concentration was resulted in loss of response. A flow rate of 0.7 mL/min was used to minimize the run time.

Protein precipitation extraction was initiated with individual tubes. Later on the method was shifted to 96 well plate format. Impact of different solutions and their concentration on recovery of analytes was monitored and the final optimized conditions are depicted in Section 2.6. During the optimization of chromatographic conditions and extraction procedure, more emphasis was given to improve the sensitivity and recovery. No significant matrix effects were observed with the proposed chromatographic and extraction conditions.

3.2 Selectivity

Selectivity of the method in rat K2 EDTA plasma was evaluated in six individual matrix lots along with one lipemic and one hemolytic lot. Peak responses in blank lots were compared against the response of spiked LLOQ and negligible interference was observed at the retention time of analytes and ISTD. Figs. 4 and 5 demonstrate the selectivity of the method with the chromatograms of blank plasma and LLOQ sample respectively.

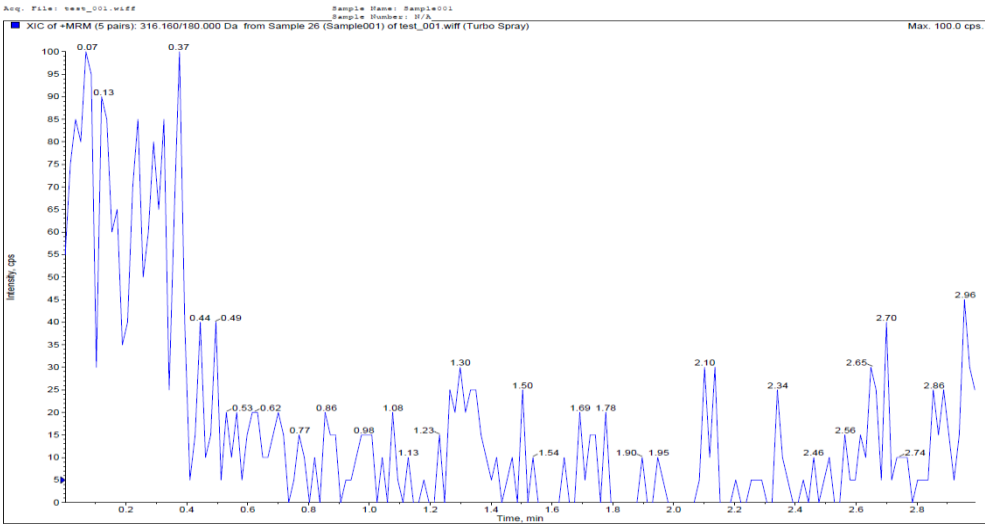

Fig 4: Blank Plasma

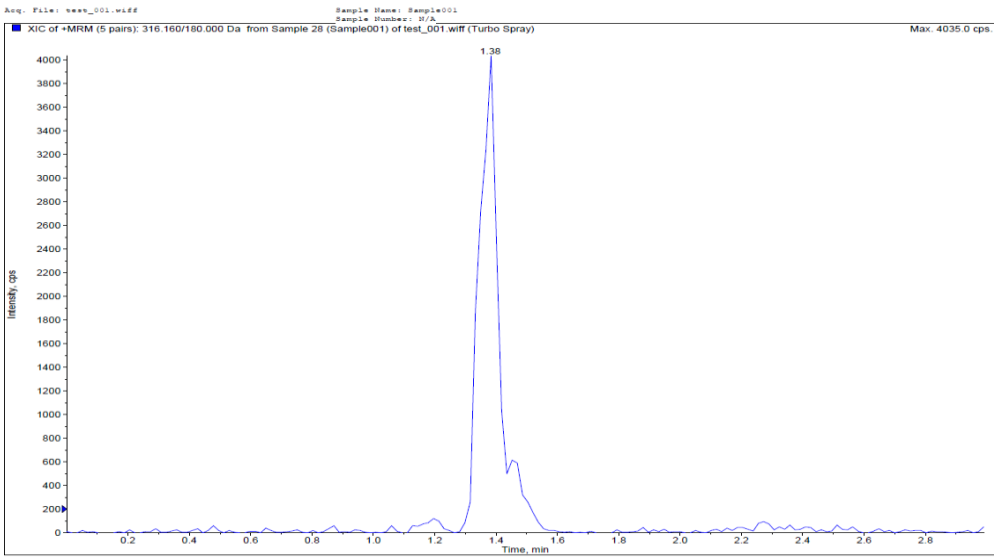

Fig 5: LLOQ

3.3 Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to ISTD versus the nominal concentration (x) of analyte. Calibration curves were linear from 0.997 to 249.259 ng/mL with r values more than 0.9990. The r values were calculated from three intra and inter day calibration curves using weighted (1/X2) quadratic regression analysis. The observed mean back calculated concentrations with accuracy (% Nominal) and precision (%CV) are presented in Table 1. The lower limit of quantitation (LLOQ) for determination of analytes was found to be 0.997 ng/mL. At LLOQ (n = 6) accuracy (% Nominal) was 97.5% with a %CV of 5.2%.

Table 1: Summary of Calibration Standards.

Analyte	Nominal (ng/mL)	Mean (ng/mL)	%CV	% Nominal
Saxagliptin	0.997	1.0090	1.8	101.2
	1.994	1.9583	3.5	98.2
	4.985	4.7877	2.2	96.0
	9.970	10.4603	1.8	104.9
	19.941	20.0760	1.8	100.7
	49.852	49.1653	2.8	98.6
	99.704	99.9837	1.2	100.3
	199.407	199.8463	0.3	100.2
	249.259	248.8497	1.0	99.8
%CV, percent coefficient of variation;				
a Mean of 3 replicates at each concentration				

3.4 Precision and accuracy

Precision and accuracy was evaluated using three intra and inter day precision and accuracy runs, with each batch consisting of six replicates of quality control samples at four concentration levels (LLOQ, LQC, MQC and HQC). The intra batch precision was between 2.5 to 8.8 % with % Nominal between 89.9 to 101.2. The inter batch precision was between 3.6 to 10.5 % with % Nominal between 101.3 to 103.6 Results of precision and accuracy are presented in Table 2.

Table 2: Intra batch and inter batch precision and accuracy.

QC level	Nominal conc. (ng/mL)	Intra Batch [a]			Inter Batch[b]		
		Mean Conc Found (ng/mL)	% CV	% Nominal	Mean Conc Found (ng/mL)	% CV	% Nominal
LLOQQC	0.997	0.9722	5.2	97.5	0.9807	5.0	98.4
LQC	2.792	2.5092	8.8	89.9	2.8277	10.5	101.3
MQC	109.674	110.9575	2.5	101.2	113.6462	3.6	103.6
HQC	189.437	178.1963	3.7	94.1	192.1321	6.1	101.4
%CV, percent coefficient of variation. Conc., Concentration							
a 6 replicates at each concentration.							
b 18 replicates at each concentration							

3.5 Matrix effect

Co-eluting matrix components can suppress or enhance the ion- ization but might not result in a detectable response in matrix blanks due to selectivity of the MS detection, however they can affect the precision and accuracy of the assay. Therefore the potential for variable matrix related ion suppression was evaluated in six independent sources (containing one hemolytic and one lipemic lot) of rat plasma, by calculating the IS normalized matrix factor. The mean IS normalized matrix factor was ranged between 0.9431 and 0.9593 with a %CV of 4.1 to 1.5 as shown in Table 3.

Table 3: Matrix Effect

Lot #	LQC			HQC		
	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	0.918	1.010	0.909	0.988	1.015	0.974
2	0.960	1.017	0.944	0.992	1.023	0.969
3	0.947	1.042	0.909	0.996	1.024	0.972
4	0.977	1.031	0.947	0.955	1.009	0.947
5	0.948	1.012	0.937	0.979	1.031	0.949
6	1.033	1.019	1.013	0.959	1.016	0.943
Mean	-		0.9431	-		0.9593
SD			0.03829			0.01397
% CV			4.1			1.5
N			6			6

MF: Matrix Factor

3.6 Extraction recovery and dilution integrity

The extraction recovery of analytes from EDTA plasma was determined by comparing the peak responses of plasma samples (n= 6) spiked before extraction with that of plasma samples spiked after extraction. The recovery was found to be 95.1%, 111.8% and 107.4% at LQC, MQC and HQC levels respectively. The mean recovery was found to be 104.74% with %CV of 8.3%, as shown in Table 4. For Internal standard the recovery was found to be 108.5%.

Dilution integrity experiment was carried out at 3 times the ULOQ concentration. After 1/10, 1/20 and 1/50 dilution the mean back calculated concentration for dilution QC samples was within 85–115% of nominal value with a %CV of ≤0.9 as shown in Table 5.

Table 4: Recovery

Analyte		A	B	% Recovery	Mean Recovery	% CV
Saxagliptin	LQC	5053.8	5317.0	95.1	104.74	8.3
	MQC	243371.5	217708.2	111.8		
	HQC	392920.3	365914.8	107.4		
Sitagliptin		33329.1	30711.7	108.5	-	-
A: Mean Peak response of Extracted Samples						
B: Mean Peak response of un Extracted Samples						

Table 5: Dilution Integrity

Dilution Factor[a]	% Nominal	% CV
1/10	94.6	3.6
1/20	95.5	0.9
1/50	99.6	2.0

a: Six replicates at each dilution factor

3.7 Stability

Stability evaluations were performed in both aqueous and matrix based samples. The stock solutions were stable for a period of 7 h at room temperature. Stock dilutions in 50% acetonitrile were stable up to 21 h 20 min at room temperature. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples).

The analyte was stable up to 4 h on bench top at room temperature and over 8 freeze-thaw cycles. The processed samples were stable up to 35 h 34 min in autosampler at 4^0C. Reinjection reproducibility is done for 22 h 49 min.

The long-term matrix stability was evaluated at both -20^0C and -50^0C over a period of 15 days. No significant degradation of analytes was observed over the stability duration and conditions. The stability results presented in Table 6 were within 85-115%.

Table 6: Stability Data

Stability	QC Level	A	% CV	B	%CV	% Change
Bench Top Stability at room Temperature (4 hrs)	LQC	2.5608	3.4	2.6922	3.8	-4.9
	HQC	179.1192	0.6	176.6337	6.0	1.4
Freeze-thaw (after 8 cycle)	LQC	2.4350	2.5	2.6774	3.0	-9.1
	HQC	181.5877	3.4	193.1057	1.2	-6.0
Auto sampler stability (35 hrs 34 min)	LQC	2.5777	4.7	2.6922	3.8	-4.3
	HQC	177.0305	3.7	176.6337	6.0	0.2
Long term stability for 90 days (below -20^{0}C)	LQC	2.6598	9.4	2.5092	8.8	6.0
	HQC	185.9143	2.9	178.1963	3.7	4.3
Long term stability for 90 days (below -50^{0}C)	LQC	2.6165	9.4	2.5092	8.8	6.0
	HQC	186.6725	2.9	178.1963	3.7	4.3

A: Mean concentration of stability samples B: Mean concentration of comparison samples.

4. CONCLUSION

A rapid, sensitive, high throughput and accurate liquid chromatography with electrospray ionization tandem mass spectrometry method was developed for determination of Saxagliptin in rat plasma with short chromatographic run time of 3.0 min. The method offers high selectivity with a LOQ of 0.997ng/mL. The extraction method utilizes a low sample volume of 100µL and shown consistent and reproducible recoveries for analyte and ISTD with minimum plasma interference and matrix effect. The validated method can be successfully used to a clinical and tox studies. Use of Sitagliptin as an ISTD will not compromise the accuracy of analytical results. The high throughput method can reduces overall processing time and allowing to process and analyze more than 180 samples in single time.

5. ACKNOWLEDGEMENTS

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