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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TENELIGLIPTIN HYDROBROMIDE HYDRATE

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ABSTRACT
To develop simple, accurate, precise, sensitive and rapid LC-MS method for the quantification of Teneligliptin in human plasma using Repaglinide as internal standard (IS). In LC-MS, estimation of Teneligliptin was carried out by using Waters Acquity UPLC system, using a Hypersil Gold C_{18} column (50 mm×3.0 mm, 1.9 µm; Thermo scientific, India) and with mobile phase 0.1% formic acid in Milli-Q water: Acetonitrile (20:80), at a flow rate of 0.5 ml/min with injection volume was 2 µL. The total chromatographic run time was 5.50 min. The analytes and IS were extracted by Solid Phase Extraction Technique. Retention time of Teneligliptin and Repaglinide was found to be 3.98 and 3.72 min. The quantification of Teneligliptin was performed in a positive electro spray ionization mode and multiple reactions monitoring (MRM) the m/z value obtained for Teneligliptin is 427.2→243.2 and for Repaglinide is 453.2→ 230.2. LC-MS method was found to be linear over the range of 1-1000 µg/ml for Teneligliptin with r^2>0.998. The method has been validated for linearity, accuracy and precision, recovery, matrix effect and Dilution integrity and stability.

KEYWORDS: Teneligliptin, LC-MS, Bioanalytical Method, validation.

1. INTRODUCTION[1-3]
Teneligliptin is an anti-diabetic drug in the class of DPP-4 inhibitors. It's a prescription medicine used along with diet and exercise to improve blood sugar (glucose) control in adults with type 2 diabetes. IUPAC name of the drug is {(2S, 4S)-4-[4-(3-methyl-1- phenyl-1H-pyrazol-5-yl) piperazin-1yl] pyrrolidin-2-yl} (1, 3-thiazolidin-3-yl) methanone. The molecular formula of the drug C22H30N6OS. The dug is having a molecular weight of 426.58g/mol.

Teneligliptin (Trade name Tenelia) is a pharmaceutical drug for the treatment of type 2 diabetes mellitus. It is approved for use in Japan, Korea & India. It belongs to the class of anti-diabetic drugs known as Dipeptidyl peptidase-4 inhibitors or "gliptins".

Teneligliptin was launched in Japan in 2012 by Mitsubishi Pharma for the treatment of type 2 diabetes mellitus. Teneligliptin is a novel oral drug which reduces the blood glucose levels in type - 2 diabetes patients. Teneligliptin is known as a member of the class of drugs which inhibits the enzyme Dipeptidyl-peptidase -4 enzyme (DPP-4).

.Incretin hormones like glucagon like peptide -1(GLP-1) and glucose dependent Insulinotropic Polypeptide (GIP) are released from the intestine after a meal. Reduction of blood glucose levels can be achieved by increasing the insulin production by the help of GLP-1 and GIP. Dipeptidyl peptidase -4, (DPP-4) can be inhibited by Teneligliptin and enhances the activity and levels of GLP-1 and GIP in the blood.

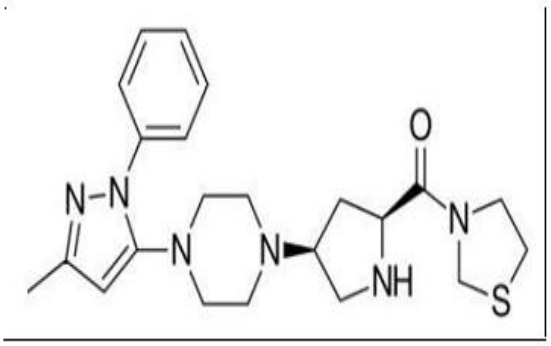

Figure 1: Structure of Teneligliptin

2. MATERIALS AND METHOD[4-14]

2.1 Instrumentation

Liquid Chromatographic Mass spectroscopy for quantitative estimation of Teneligliptin LC-MS instrument having Waters Acquity UPLC system with Hypersil Gold C18 column (50 mm×3.0 mm, 1.9 µm) was used. The analyte and IS were separated by using the mobile phase 0.1% formic acid in Milli-Q water and Acetonitrile by gradient delivered with a flow rate of 0.5 mL/min. The injection volume was 2µl and the total chromatographic run time was 5.50 min. The analytes and IS were detected using a Waters XEVO TQ mass spectrometer (Waters corporation, Milford, USA) equipped with Z spray source. The precursor to product ion transitions for both analytes and IS were as follows: Teneligliptin m/z 427.2→243.2, Repaglinide m/z 453.2→ 230.2 with dwell time 100 ms. Data acquisition and calculations were performed using Analyst software, version 4.1.2.

2.2 Chemicals and reagents

The pure drug form of Teneligliptin is collected from Precise Chemipharma Pvt. Ltd, Maharashtra. Repaglinide (IS) is collected from Torrent pharmaceuticals Ltd. Methanol (HPLC grade), Acetonitrile (HPLC grade), Milli-Q water, Formic acid, DMSO were purchased from Merck, Rankem. K3EDTA and Sodium Heparinized human plasma.

2.3 Chromatographic conditions

Chromatographic separation was performed on Hypersil Gold C18 column (50 mm×3.0 mm, 1.9 µm) using mobile phase 0.1% formic acid in Milli-Q water: Acetonitrile (20:80), at a flow rate of 0.5 ml/min with injection volume was 2 µL. The total chromatographic run time was 5.50 min. The column oven temperature was set at 40.0 ± 3.0°C. Retention Time of Teneligliptin was 3.98 and Repaglinide was 3.72.

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)
Teneligliptin	427.2	243.2	100	65	28	15
Repaglinide	453.2	230.2	100	36	25	22

2.5 Preparation of calibration standards and quality control samples

Standard stock solutions of Teneligliptin and Internal standard (Repaglinide) were prepared by dissolving 10 mg of both the Drugs and Transfer it in 10ml volumetric flasks. Add about 5.0 ml of Methanol and sonicate to dissolve. Dilute up to mark with Methanol to give a final concentration of 1mg/mL.

For spiking stock solutions of Teneligliptin and Internal standard (Repaglinide) transfer the calculated volume 0.5ml of solution in 10 ml volumetric flask and dilute up to 10 ml mark with Methanol to make conc. 50.00 µg/ml.

Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with the working solutions (5%) prepared from stock solution.

2.6 Sample Preparation

2.6.1 Aliquot 0.2 ml of sample into pre-labelled tubes.

2.6.2 Add 50 µl of Mixed ISTD Dilution (Repaglinide) to all samples except of STD BL sample and vortex for about 30 seconds.

2.6.3 Add 500 µl of Extraction buffer to all solutions and vortex for about 30 seconds.

2.6.4 Arrange the required number of pre-labelled Strata-X 30 mg, 1 ml extraction cartridges on EZYPRESS 48-48 Position Positive Pressure Processor or on solid phase extraction manifolds.

2.6.5 Condition the cartridges with 1 ml methanol followed by 1 ml water.

2.6.6 Load about 700 µl of prepared samples on conditioned cartridges carefully.

2.6.7 Wash the cartridges with 1 ml water and 1 ml 10% methanol in water twice. Dry the cartridges for 2 minutes by applying positive pressure at maximum flow rate or by applying full vacuum.

2.6.8 Elute the contents from cartridges with 1 ml methanol into pre-labelled tubes and evaporate the samples to dryness under nitrogen gas at about 40±5°C.

2.6.9 Reconstitute the dried samples with 100 µl mobile phase and vortex for about 30 Seconds.

2.6.10 Transfer samples into pre-labeled auto-sampler vials and inject by using LC-MS.

2.7 Method validation

The bioanalytical method was validated according to the USFDA guidelines. The method was validated for selectivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity, Stability.

System suitability experiment is performed to check whether system is suitable or not to carry out experiments. System suitability was performed by injecting six consecutive injections using aqueous MQC at least once in a day. Acceptance criteria: % CV of retention time (tR) and area ratio should be ≤ 4.00%.

System performance experiment is carried out to check that system is capable to perform as per its predetermined specifications or not. For system

performance extracted STD BL, ULOQ and LLOQ samples are processed and injected for determination of % interference in blank and % carryover effect of system performance is carried out before each batch experiment. Acceptance criteria: Peak area of analyte should be more than or equal to 5.0 times of LLOQ sample when compared to 1st acquired STD BL(ULOQ). Carryover observed in both STD BL injected after ULOQ should be ≤ 20.0% for analyte and ≤5.0% for ISTD response compared to analyte and ISTD response respectively of LLOQ sample. % Interference observed in first acquired STD BL (before ULOQ) is > 20.0% for analyte and > 5.0% for ISTD in this case repeat same experiment by using separately processed samples.

Specificity is performed by using 10 different plasma lots among which 7 lots of normal plasma having K3EDTA as anticoagulant, 1 lot of hemolyzed plasma, 1 lot of lipemic plasma and 1 lot of plasma having sodium heparin as anticoagulant. Sample processed: STD BL and LLOQ. Compare response of interfering peak at Rt of analyte and ISTD in STD BL against response of extracted LLOQ sample. Acceptance criteria: Response of interfering peak at Rt of analyte should be ≤20.0% and at Rt of ISTD should be ≤5.0% response of ISTD of respective LLOQ samples. At least 80% of matrix lots (excluding hemolyzed, heparinized and lipemic matrix lots) with intended anticoagulant should be within aforementioned acceptance criteria.

Recovery is a measure of efficiency at which an analytical method recovers the analyte through sample processing step. Recovery should be performed by comparing analytical results for extracted samples at three concentrations(low, medium and high) and three replicates with un-extracted standards that represents 100% recovery. Recovery of analyte need not to be 100%, but extent of recovery of an analyte and of ISTD should be consistent, precise, and reproducible. Acceptance criteria: % CV at each level (high, medium and low) over all % CV should be ≤ 15.00%.

Matrix Factor the ratio of analyte response in presence of matrix ions to in absence of matrix ions. Matrix effect: An alteration of analyte response due to matrix component in sample. Matrix effect is evaluated by calculating matrix factor. The matrix factor is determined by comparing analyte response in presence of matrix with that in absence of matrix. Matrix factor can be normalized by using an internal standard.

Matrix factor is performed by processing n ≥5 of each extracted (samples with matrix ions) and aqueous (samples without matrix ions) samples at high and low concentrations by using six different matrix lots obtained from different sources. Acceptance criteria:
% CV of ISTD normalized factor at each level (high, medium, low) should be ≤15.00%.

Calibration curve (standard curve) is the relationship between the response of the instrument and known concentrations of the analyte(s). Calibration curve should be prepared for each analyte(s) in the sample. A sufficient number of standards should be used in order to properly define the relationship between concentration and response. A calibration standard should be prepared in the same biological matrix same as that of the study samples, by spiking with known concentrations of the analyte(s). A calibration curve should be comprised of a "blank matrix" (matrix processed without analyte and internal standard), a "zero standard" (blank matrix processed only with internal standard) and six or more calibration standards covering the expected range, including the LLOQ and ULOQ.

LLOQ should cover at least 4-5 half-life of the reported Cmax. ULOQ should cover the expected Cmax value. When no reference is available, it should be decided on basis of development studies results. Calibration curve must contain minimum six calibration standards. Selection of calibration standard expressed as multiple of LLOQ and percent of ULOQ are as follows:

Table 1: Protocol for calibration level selection

Code	9 std calibration curve
STD-1/ CS-1	LLOQ
STD-2/ CS-2	2 LLOQ
STD-3/ CS-3	5% ULOQ
STD-4/ CS-4	10% ULOQ
STD-5/ CS-5	20% ULOQ
STD-6/ CS-6	40% ULOQ
STD-7/ CS-7	60% ULOQ
STD-8/ CS-8	80% ULOQ
STD-9/ CS-9	100% ULOQ

Precision and Accuracy Intra-day precision and accuracy were determined by analyzing six replicate analysis of each quality control (LLOQ, LQC, MQC and HQC) samples of 3 different batches on same day. Inter-day precision and accuracy were determined by analyzing six replicate analysis of each quality control (LLOQ, LQC, MQC and HQC) samples of five different batches. The acceptance criteria included accuracy with in ±15% deviation from the nominal values, except the LLOQ where it should be ±20% and a precision of ±15% coefficient of variance (%CV), except for LLOQ, where it should be ±20%.

NOTE: Three precision and accuracy batch are performed for between run precision and accuracy and one for different analyst, analyst and column.

Dilution Integrity of Stock Solutions (DISS) is performed to demonstrate that dilution of samples should not affect accuracy and precision. If applicable dilution integrity should be demonstrated by spiking matrix with an analyte concentration above ULOQ and diluting this sample with blank matrix(at least 5-10 times per dilution

factor). Dilution integrity experiment is performed by using n ≥ 5 samples of dilution integrity by spiking matrix with an analyte concentration 4-5 times above ULOQ and diluting this sample with blank matrix (at least 5-10 times per dilution factor). For DI Prepare drug intermediate of 1000 µg/ml from drug stock solution. This prepared solution called as DISS (1/10). 5% spiking of DISS (1/10) solution spiked into blank plasma and final concentration of spiked solution became µg/ml (AUL QC). AUL QC further 10 times diluted with plasma gives 5000ng/ml which is 5 times of ULOQ and analysed with 3 precision and accuracy batch. Acceptance Criteria: % CV of dilution integrity sample should be ≤ 15.00% and accuracy should be within 15.00%.

Hemolytic and Lipemic effect (HLE) is perform to demonstrate that hemolyzed and lipemic plasma should not affect accuracy and precision. HLE should be performed by spiking of SS HQC and SS LQC in hemolytic and lipemic plasma. It is analysed along with freshly spiked calibration curve STD and quality control samples in normal K3EDTA plasma. Acceptance Criteria: % CV of HLE sample should be ≤ 15.00 % and accuracy should be within 15.00%.

Auto-sampler Reinjection Reproducibility (ASRR) performed to check reproducibility of auto-sampler. Hence, for ASRR inject previously accepted precision and accuracy batch. Acceptance Criteria: For ASRR, HQC, MQC and LQC samples should be within ± 15% mean accuracy and for LLOQ it should be ± 20% mean accuracy.

Short Term Stock Solution Stability (STSS):Stability of drug and ISTD stock solution should be evaluated for at least 06 hours. For STSS stock solutions are stored in refrigerator for minimum of 06 hours and after stability period retrieve it and make ULOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ and ISTD dilution.

Long Term Stock Solution Stability (LTSS): Stability of drug and ISTD stock solution should be evaluated for relevant time period. For LTSS stock solutions are stored in refrigerator for 20 days and after stability period retrieve it and make ULOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ and ISTD dilution.

Short Term Working Solution Stability (STWSS): Stability of drug and ISTD working solution should be evaluated for at least 06 hours. For STWSS working solution are stored in refrigerator for minimum of 06 hours and after stability period retrieve it and make ULOQ, LLOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ, LLOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ, LLOQ and ISTD dilution.

Long Term Working Solution Stability (LTWSS): Stability of drug and ISTD working solution should be evaluated for relevant time period. For LTWSS working solution are stored in refrigerator for 20 days and after stability period retrieve it and make aqueous ULOQ, LLOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ, LLOQ and ISTD dilution.

Bench Top Stability (BT) (Short Term Stability of Analyte in Matrix):It should be performed at higher quality control and lower quality control level for six replicates. Prepare spiked sample of HQC and LQC and stored at room temperature for a specific time period. Generally, time period is about time required from spiking of sample to transfer in to vials. Use freshly spiked calibration curve and quality control standard for determination of stability samples. For BT kept spiked HQC and LQC for 06 hours at room temperature. BT stability samples were analyzed along with freshly spiked CCs and freshly prepared QCs 6 replicates of each LQC and HQC samples as per procedure.

Stability of Dry Extract (DE): DE experiment was carried out whenever sample processing involves evaporation step. DE stability was conducted by using previously processed and dried stability samples. Freshly spiked replicates of each LQC and HQC samples were prepared and processed as per sample preparation procedure. After drying, dry extract stability samples were stored at -20 ± 5ºC for a period of at least 24 hours or as per requirement. DE stability samples were analyzed along with freshly spiked CCs and freshly prepared QCs 6 replicates of each LQC and HQC samples as per procedure.

Freeze and Thaw Stability: QC samples (at high and low level) are stored and frozen in freezer at intended temperature and thereafter thawed at room or processing temperature. After complete thawing, samples are refrozen again applying same conditions. At each cycle, samples should be frozen for at least 12 hours before they are thawed. It is perform to demonstrate that accuracy and precision is not change upon freezing and thawing cycle. Freeze and thaw stability experiment is performing by processing n ≥ 5 sample (at high and low level) of freeze thaw stability along with freshly spiked calibration curve and quality control sample. Storage temperature: -20 ± 5°C and -78 ± 8°C.

3 RESULT AND DISCUSSION

3.1 LC-MS METHOD DEVELOPMENT AND VALIDATION OF TENELIGLIPTIN IN BULK FORM.

3.1.1 System Suitability

% CV of Analyte tR, ISTD tR and Area Ratio for drug was observed 0.38, 0.202 and 0.4247 respectively for

Teneligliptin which is not more than 4.00% as per acceptance criteria.

3.1.2 System Performances

% Carryover in STD BL2 and STD BL3 was found to be 0.61% and 0.4% respectively at tR of teneligliptin which is not more than 20% and % carry over in STD BL2 and STD BL3for Repaglinide were 0.006 and 0.008% respectively at Rt of which is not more than 5%. So results are accepted as per acceptance criteria.

% Interference in STD BL was found to be 1.63% at tR of Teneligliptin which is not more than 20% and % carry over STD BL1 was found to be 0.011% at Rt of Repaglinide which is not more than 5%. So results are accepted as per acceptance criteria.

3.1.3 Specificity or Selectivity

% Interference at retention time of drug & ISTD at LLOQ was found to be 0.13 to 2.03% & 0.001 to 0.010% respectively Teneligliptin and Repaglinide which is within acceptance criteria. All plasma lots are not interfering at tR of analyte and ISTD, so these plasma lots can be used for further validation experiments.

3.1.4 Linearity

The nine point calibration curve showed good linearity in the range of 1-1000 ng/ml, for Teneligliptin with correlation coefficient (r2) of 0.998.% Mean accuracy of concentration of calibration curve standards of 3 P & A run was found within ± 15% and % CV of 3 P & A run was found to be < 15%.

Table 2: Summary of Calibration Standards.

STD ID	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal Conc.	1 ng/mL	2 ng/mL	10 ng/mL	100 ng/mL	200 ng/mL	400 ng/mL	600 ng/mL	800 ng/mL	1000 ng/mL
Mean	0.96	1.88	9.61	95.91	195.79	390.4	587.48	743.4	939.41
% CV	8.45	5.96	4.98	3.38	3.59	1.39	10.61	1.08	3.36
Mean Accuracy	96.25	94.14	96.13	95.91	97.89	97.6	97.91	92.92	93.94

3.1.5 Precision and Accuracy

Intra-run precision for all high, middle and low quality control samples was found to be ≤ 1.50% for Teneligliptin which is within acceptance limit of 15.00%. Intra-run precision for all LLOQ QC quality control sample was found to be ≤ 2.10% for Teneligliptin which is within acceptance limit of 20.00%. Intra-run accuracy for all high, middle and low quality control samples was found to be ≤ 10.08% for Teneligliptin which is within acceptance limit of ± 15.00%. Intra-run accuracy for all LLOQ QC quality control sample was found to be ≤11.8% for Teneligliptin which is within acceptance limit of ± 20.00%.

Inter-run precision for all high, middle and low quality control samples was found to be ≤ 3.72% for Teneligliptin which is within acceptance limit of 15.00%. Inter-run precision for all LLOQ quality control sample was found to be ≤ 4.02% for Teneligliptin which is within acceptance limit of 20.00%. Inter-run accuracy for all high, middle and low quality control samples was found to be ≤ 9.55% for Teneligliptin which is within acceptance limit of ± 15.00%. Inter-run accuracy for all LLOQ quality control sample was found to be ≤ 13.4% for Teneligliptin which is within acceptance limit of ± 20.00%.

Table 3: Summary of Intra-Run Precision & Accuracy

P & A ID	QC Sample	Mean	% CV	% Mean Accuracy
P & A I	LLOQ QC	0.88	2.1	88.2
	LQC	2.94	1.5	98
	MQC	364.45	0.98	91.11
	HQC	744.74	0.81	93.09
	ULOQ	952.57	1.20	95.25
P & A II	LLOQ QC	0.89	1.21	89.03
	LQC	2.89	0.39	96.42
	MQC	365.04	0.94	91.26
	HQC	737.34	0.79	92.16

	ULOQ	953.43	0.91	95.34
P & A III	**LLOQ QC**	0.91	1.43	91.08
	LQC	2.83	1.32	94.35
	MQC	365.39	0.89	91.34
	HQC	719.37	0.55	89.92
	ULOQ	895.94	1.23	89.59

Table 4: Inter-run Precision and Accuracy

P & A	**LLOQ QC**	**LQC**	**MQC**	**HQC**	**ULOQ**
	1 ng/mL	**3 ng/mL**	**400 ng/mL**	**800 ng/mL**	**1000 ng/mL**
Mean	0.866	2.886	365.838	723.64	917.755
% CV	4.022	0.411	0.876	3.723	1.875
% Mean Accuracy	86.6	96.222	91.459	90.455	91.775

3.1.6 Recovery

% Overall recovery for drug and ISTD was found to be 88.47% & 98.41% respectively for Teneligliptin and ss Repaglinide. % Overall CV of recovery for drug at HQC, MQC and LQC was found to be 5.297% for Teneligliptin which is within acceptance criteria.

Table 5: Recovery of Analyte

	HQC		**MQC**		**LQC**	
	Ex PA	**Un- Ex PA**	**Ex PA**	**Un- Ex PA**	**Ex PA**	**Un- Ex PA**
Mean	1084912	1174759	510707.8	566809.8	9284	11187.67
% CV	0.067	1.885	0.217	1.525	1.225	6.385
%Mean Recovery	92.35		90.1		82.98	
Correction factor	1.06					
% Mean Recovery with CF	97.89		95.50		87.95	
% Overall Recovery	88.47					
% Overall Recovery with CF	97.89					
% Overall CV	5.297					
Ex PA (Extracted Peak Area)						
Un- Ex PA (Un-extracted Peak Area)						

Table 6: Recovery of IS

	ISTD	
	Ex PA	**UN Ex PA**
Mean	1584703	1610207
SD	9246.277	9177.462
% CV	0.583471	0.569955
%Mean Recovery	98.41	
% Mean Rec with CF	104.31	

3.1.7 Matrix effect

Variability in ISTD normalized matrix factor, as measured by coefficient of variation was found to be 2.88% for HQC level and 2.04% for LQC level for Teneligliptin which was ≤ 15% according to acceptance criteria.

Table 7: Matrix Effect

Lot #	LQC			HQC		
	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	1	1	1	0.99	0.99	1
2	1.01	1	1.01	1	0.98	1.02
3	1	1.01	0.99	0.99	0.96	1.03
4	0.99	0.96	1.03	0.97	1	0.97
5	1	0.99	1.01	1.03	0.99	1.04
6	1	0.98	1.02	0.99	0.95	1.03
Hemolytic	1	0.96	1.04	1	0.93	1.07
Hemolytic	0.98	0.96	1.02	1.01	0.99	1.02
Lipemic	0.98	1.01	0.97	0.99	0.98	1.01
Lipemic	0.99	0.99	1	0.99	1	0.99
Mean	0.99	0.99	1.00	0.99	0.97	1.01
SD	0.020			0.029		
% CV	2.048			2.887		

MF: Matrix Factor.

3.1.8 Dilution integrity

% Mean accuracy of dilution quality control was found to be 99.55%, 97.96% and 98.91% for Teneligliptin P & A I, P & A II, P & A III respectively which is within acceptance criteria. % Mean accuracy of dilution quality control is within ± 15% and % CV of dilution quality control is within 15%, so dilution of sample does not affect precision and accuracy of method.

Table 8: Results for Dilution Integrity

P & A ID	P & A I	P & A II	P & A III
QC	DQC	DQC	DQC
	5000ng/mL	5000ng/mL	5000ng/mL
Mean	4977.87	4898.221667	4945.82
% CV	0.881939152	1.276867884	0.971248055
% Mean Accuracy	99.5574	97.96443333	98.9164

3.1.9 STABILITY

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The short term and long term stock solution and working solution stability was determined by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability, dry extract stability, freeze–thaw stability were tested at LQC and HQC levels using six replicates at each level.

Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15%) and precision (±15% CV).

Table 9: Summary of stability data of Teneligliptin in human plasma

Stability	QC Level	A	%CV	B	% CV	% Change
STSS	**ULOQ**	915.23	2.317	952.97	0.880	3.960
LTSS	**ULOQ**	917.05	1.814	963.09	0.762	4.780
STWSS	**ULOQ**	908.83	0.919	955.54	0.971	4.888
	LLOQ	0.899	1.324	0.932	1.800	3.540
LTWSS	**ULOQ**	910.67	1.480	956.49	0.873	4.790
	LLOQ	0.890	0.453	0.931	1.292	4.403
BT	**LQC**	2.838	1.374	2.866	0.941	0.976
	HQC	774.82	1.647	782.98	0.697	1.042
DE	**LQC**	2.877	1.816	2.889	0.510	0.415
	HQC	756.82	2.947	762.09	4.129	0.691

SE (5 ± 3°C)	**LQC**	2.870	1.260	2.883	0.466	0.450
	HQC	774.05	0.768	777.64	0.371	0.461
SE (Ambient Temperature)	**LQC**	2.86	2.108	2.872	1.897	0.417
	HQC	758.91	1.266	768.22	1.035	1.211
FT (-20 ± 5°C)	**LQC**	2.840	2.041	2.879	2.218	1.354
	HQC	773.87	1.065	777.44	0.780	0.459
FT (-78 ± 8°C)	**LQC**	2.885	0.371	2.894	1.580	0.310
	HQC	775.46	1.256	776.52	0.596	0.136

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

Calibration Curve

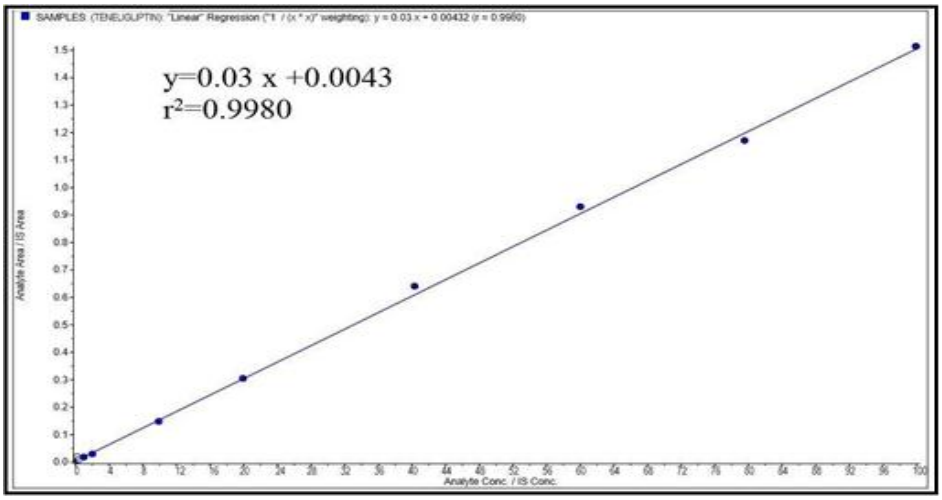

Figure 2: Calibration curve of Teneligliptin

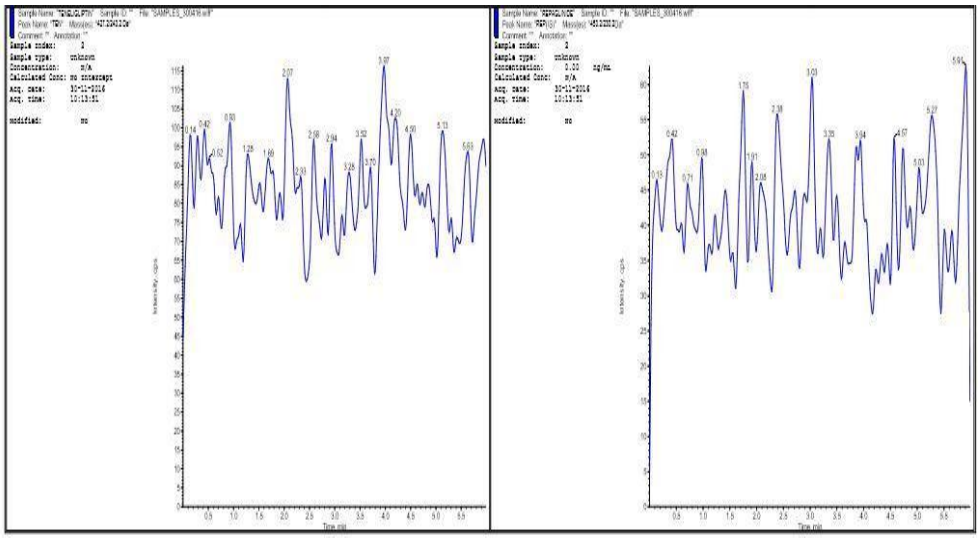

Figure 3: Representative chromatograms of Blank

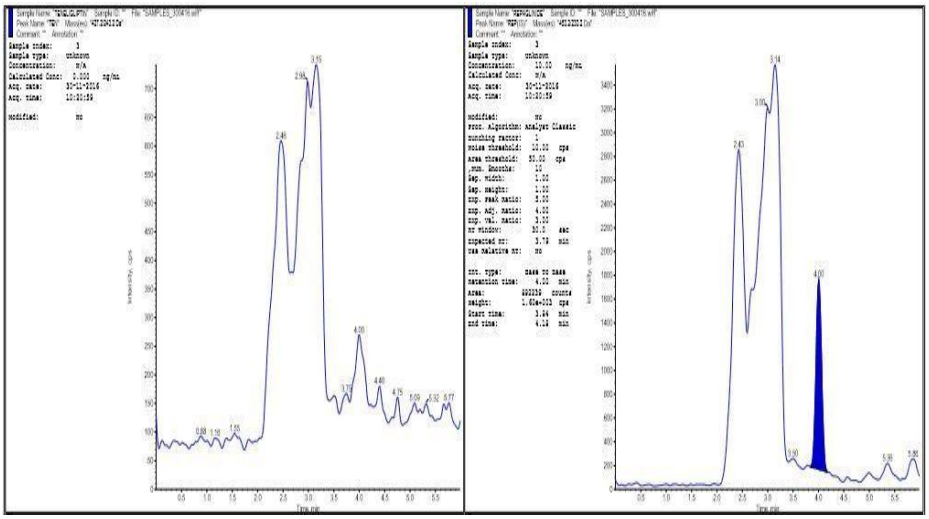

Figure 4: Representative chromatograms of Blank-IS

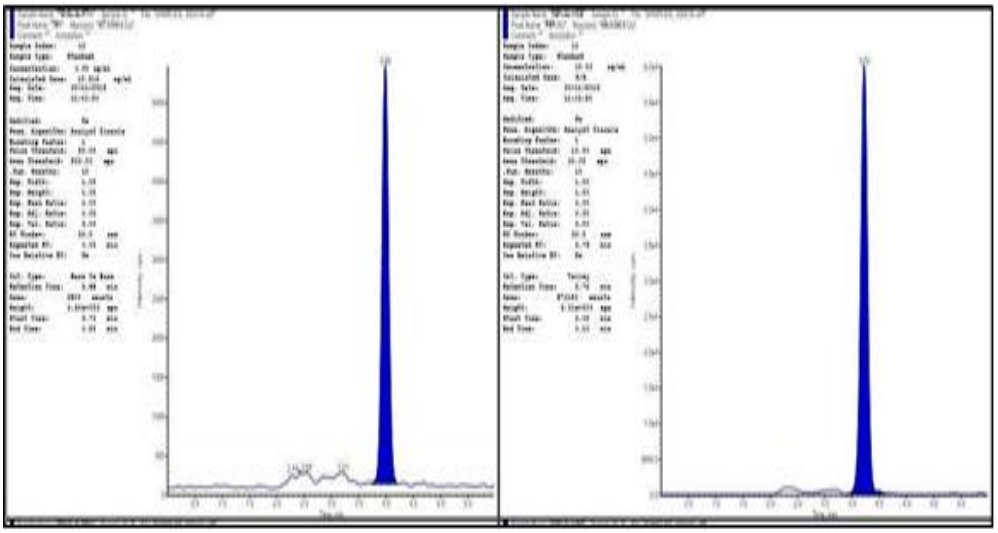

Figure 5: Representative chromatograms of STD-1

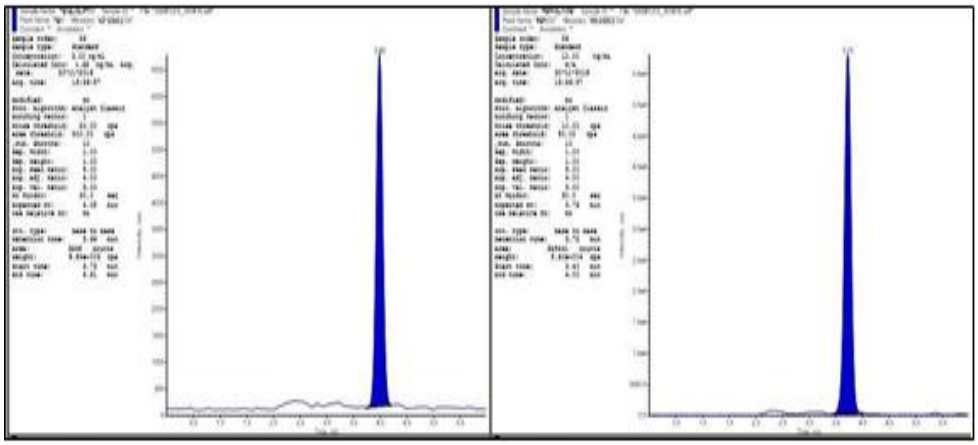

Figure 6: Representative chromatograms of STD-2

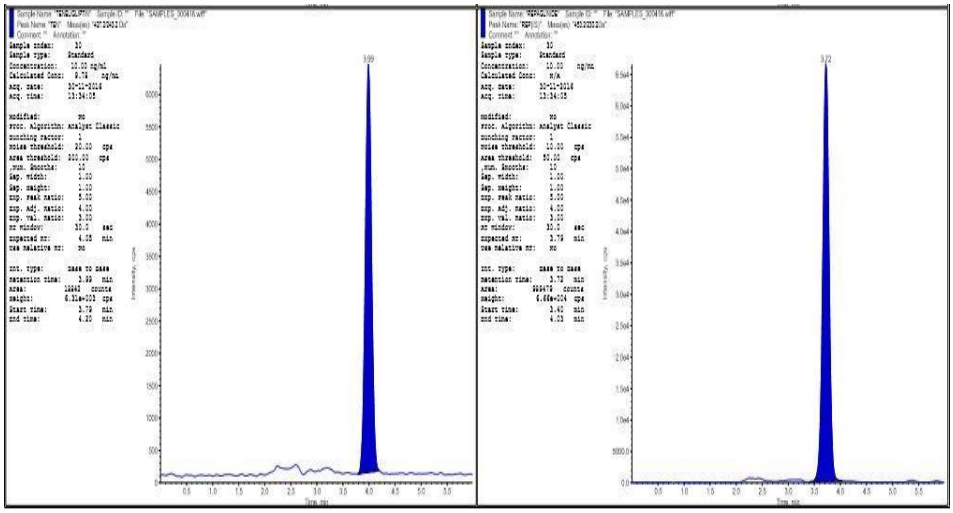

Figure 7: Representative chromatograms of STD-3

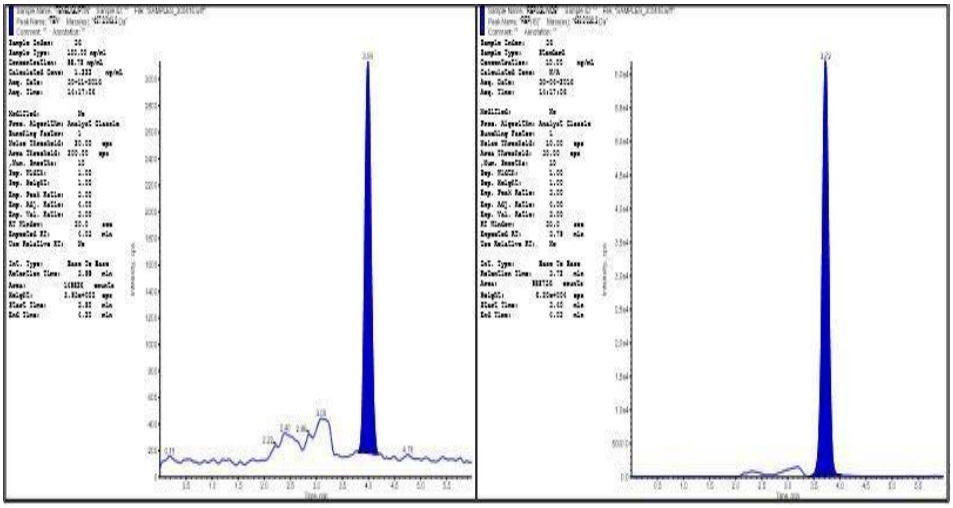

Figure 8: Representative chromatograms of STD-4

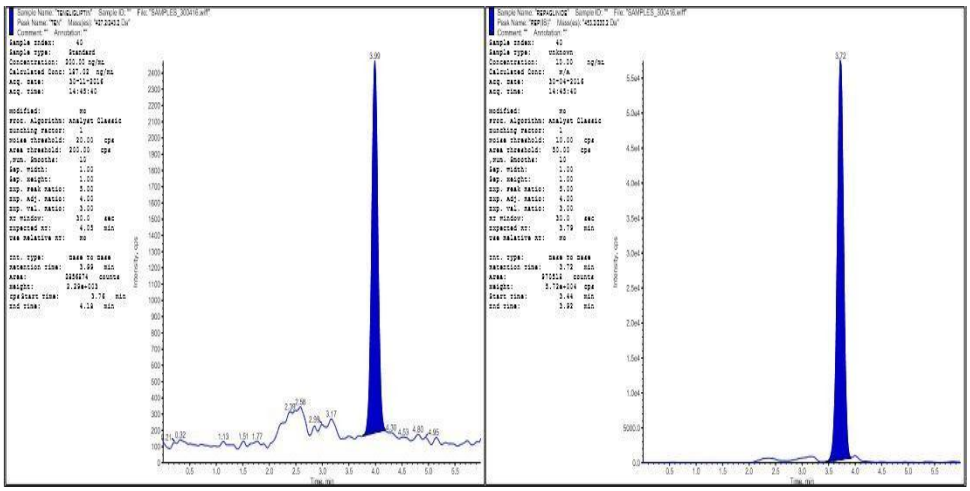

Figure 9: Representative chromatograms of STD-5

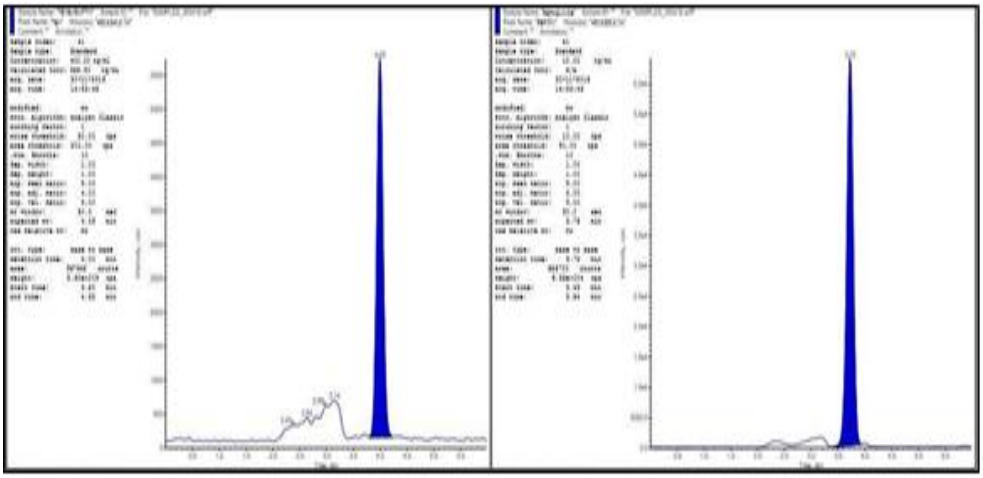

Figure 10: Representative chromatograms of STD-6

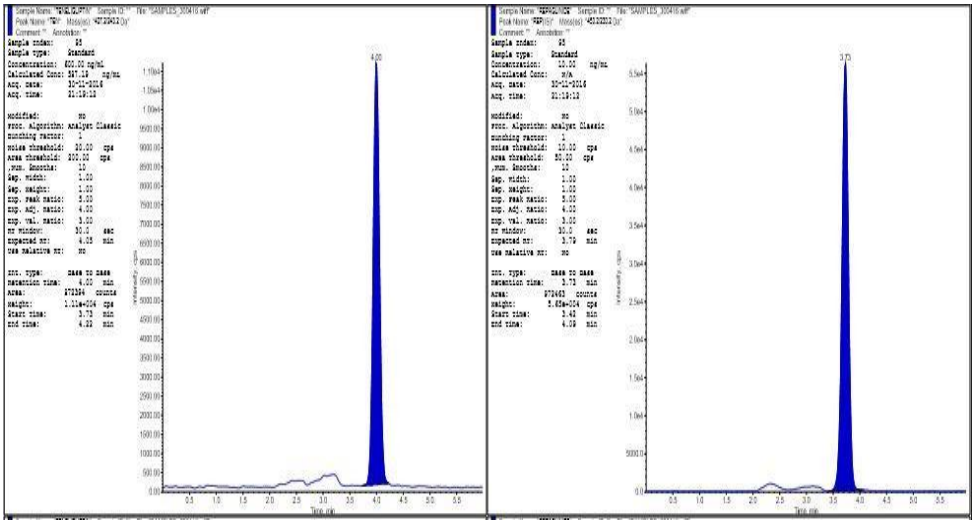

Figure 11: Representative chromatograms of STD-7

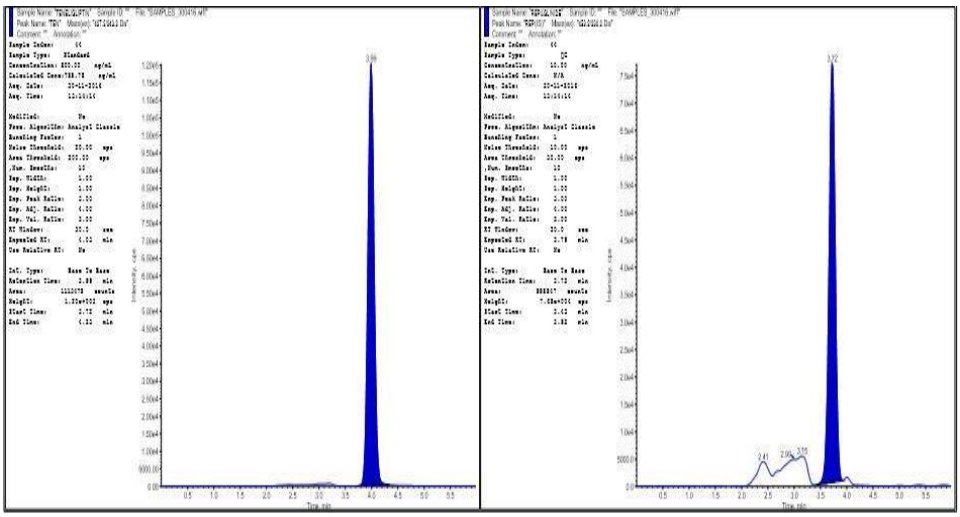

Figure 12: Representative chromatograms of STD-8

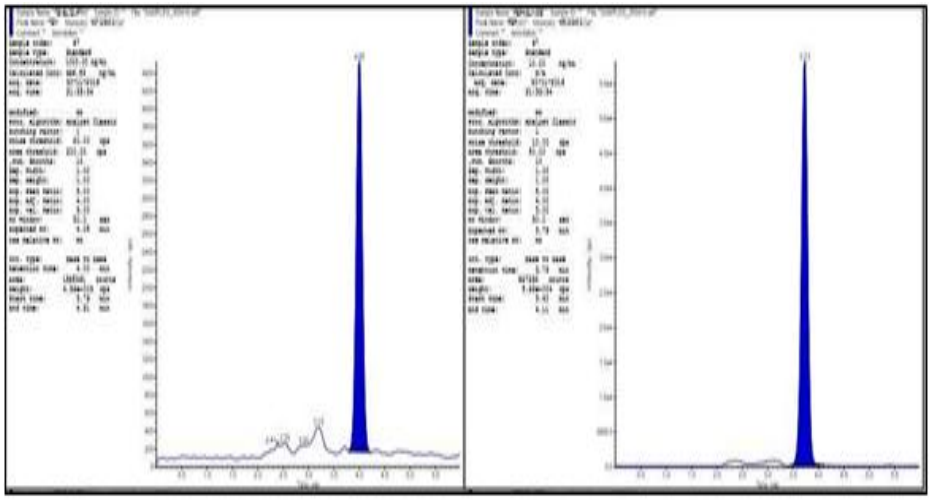

Figure 13: Representative chromatograms of STD-9

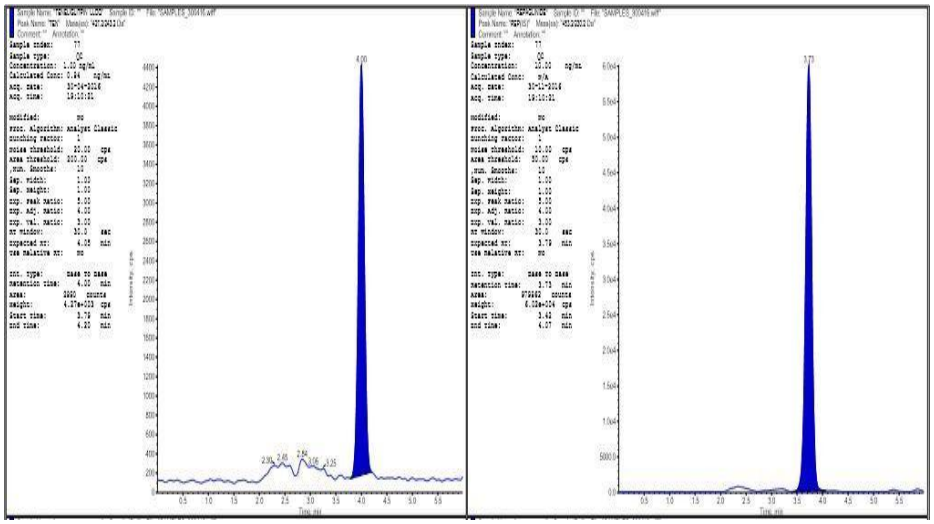

Figure 14: Representative chromatograms of LLOQ

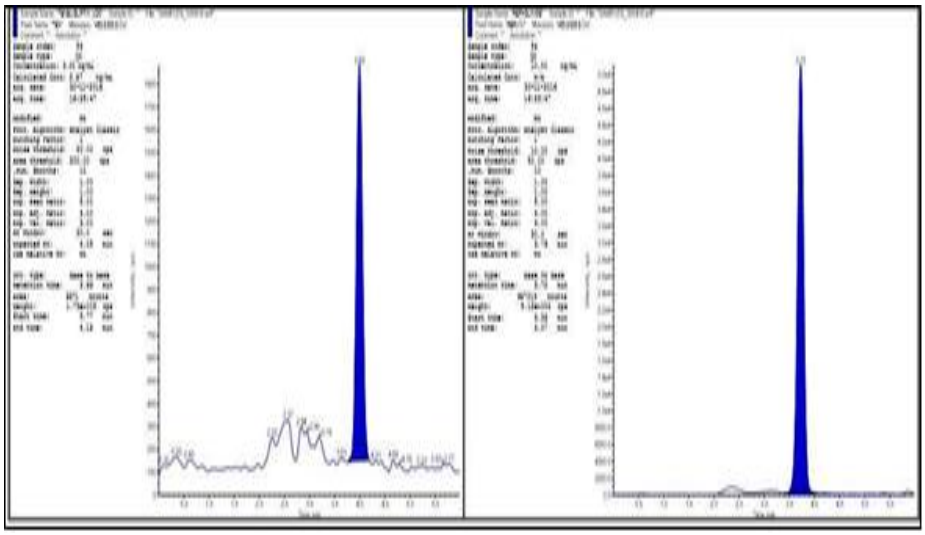

Figure 15: Representative chromatograms of LQC

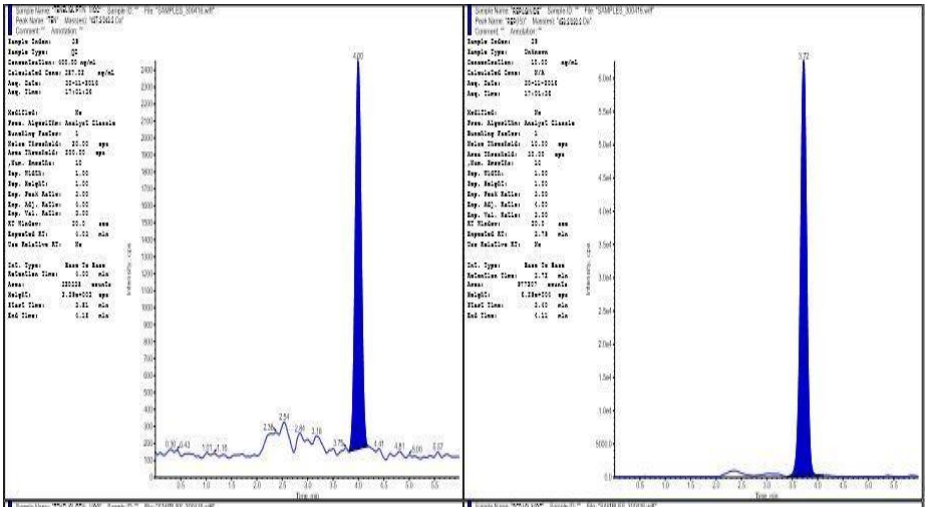

Figure 16: Representative chromatograms of MQC

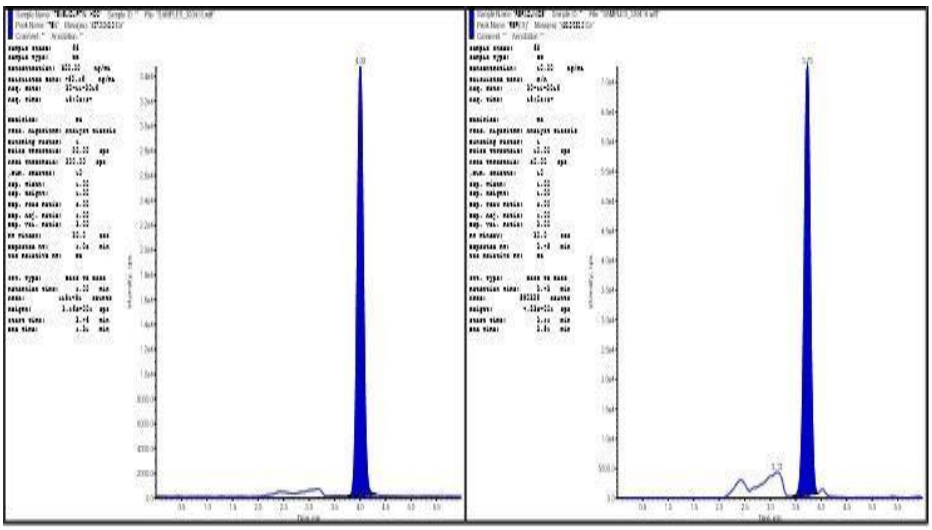

Figure 17: Representative chromatograms of HQC

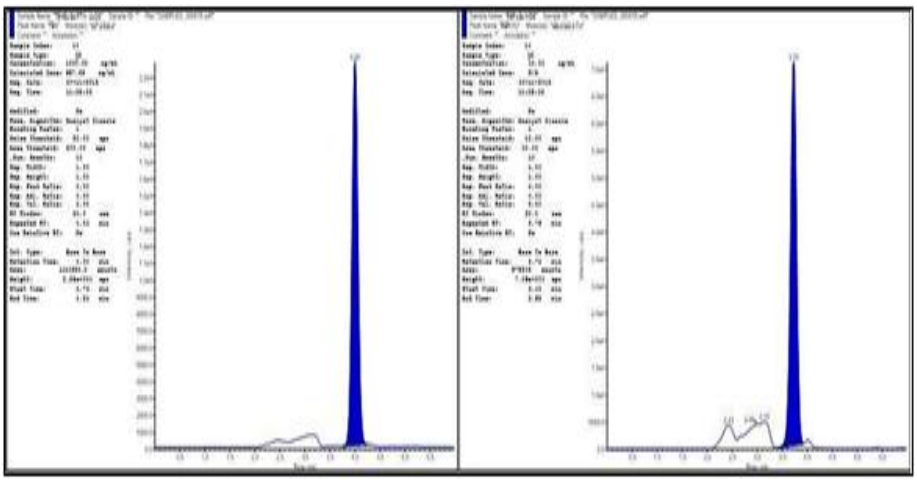

Figure 18: Representative chromatograms of ULOQ

CONCLUSION

The method is considered valid for extraction and analysis of Teneligliptin in K3EDTA human samples within investigated concentration range of 1-1000ng/mL. The validation criteria used in present study is instrument stability, sample preparation strategy, calibration, precision and accuracy, specificity and selectivity at different concentrations of both the drugs. Upon injection of spiked plasma after a valid extraction procedure into LC-MS instrument at LLOQ, specificity

and selectivity shows acceptable results which means that instrument at this lowest concentration is capable to give a reproducible result. There was no significant peak observed from endogenous compounds at retention time of analyte and internal standard which means chromatographic conditions were quite perfect for satisfactory separation within chromatographic run time (5.5 minutes).

Hence, it can be concluded that simple, rapid and sensitive reverse phase liquid chromatographic- mass spectrometric method was devised to quantify Teneligliptin in human plasma and can be successfully applied for bioequivalence studies in human subjects. The developed method is fully validated as per USFDA and EMEA guidelines compared to reported methods.

4. ACKNOWLEDGEMENTS

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