Original Research Article

International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com
OPEN ACCESS

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LINAGLIPTIN IN PLASMA THROUGH LC-MS/MS

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Received for publication: May 21, 2014; **Accepted:** June 06, 2014

Abstract: The objective of the study was to develop and validate simple, selective, specific Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) method for the determination of Linagliptin in Human Plasma. The accuracy and precision data must fulfill the requirements for the quantification of analytes in biological matrices to produce data for bioavailability, bioequivalence, etc. The separation of the analyte was carried out on Waters, X-Bridge, C18, 5µm column having 4.6×50 mm internal diameter and the mobile phase containing acetonitrile and 0.1 % formic acid (90:10 v/v) at a flow rate of 0.6 mL/min. The retention times of Linagliptin and Telmisartan (Internal Standard) were 1.45 min and 1.20 min simultaneously and the total run time was 3.0 min. Monitoring of the fragmentation of m / z 473.54 → 157.6 performed during MS/MS detection of Linagliptin and Internal Standard (I.S.) on the mass spectrometer. The overall recovery of Linagliptin and IS was 92.5 % and 89.9 % respectively. The matrix effect of Linagliptin and IS was 5.51 and 1.33 % respectively. The method was validated over the concentration range of 10ng/mL to 5000ng/mL. Multiple Reaction Monitoring (MRM) mode was used as an operating mode in the mass spectrometer. Ion spray was kept in positive mode for the detection of Analyte and IS during the production of ions. The method was validated for linearity, accuracy, precision, specificity, selectivity, inter and intraday precision, LQC, HQC.

Key Words: Linagliptin, Bioavailability, Validation, Accuracy, LC-MS/MS.

INTRODUCTION

Linagliptin, which can be described chemically as 8-[(3R)- 3- amino -1- piperidinyl] -7-(but-2-yn-1-yl) -3-methyl-1-[4methyl quinazolin-2-yl)methyl3, 7-dihydro-1H-purine-2, 6dione] is a novel oral drug which reduces the blood glucose levels in type - 2 diabetes patients. Linagliptin is known as a member of the class of drugs which inhibits the enzyme dipeptidyl-peptidase -4 enzyme (DPP-4) [5,6]. Incretin hormones like glucagonlike peptide -1(GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are released from the intestine after a meal [2]. 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 Linagliptin and enhances the activity and levels of GLP-1 and GIP in the blood [7, 8]. The plasma half-life of GLP-1 is less due to its proteolytic degradation by DPP-4, hence it is necessary to increase the plasma half-life of GLP-1 and it can be achieved by inhibiting the DPP-4 by Linagliptin [3, 4]. Glitazones increase sensitivity of liver, muscle, fat to insulin [1].

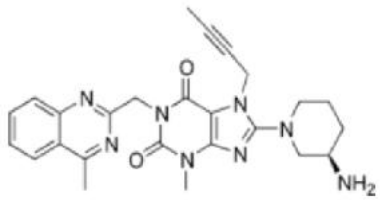

Figure 1: Structure of Linagliptin

MATERIALS AND METHODS

Reagents and Materials

Linagliptin was purchased from Manus Aktteva Biopharma LLP, Ahmadabad, India. Batch number 1052 / 081. Methanol: HPLC grade (Ranbaxy Labs Ltd., India), Acetonitrile gradient: HPLC grade (Ranbaxy Labs Ltd., India), Milli-Q water: HPLC grade, Control Human plasma: Kavery Blood bank, Rastrapathi road, Hyderabad, India.

Instrumentation

Table 1:

System	Agilent 1100 Quaternary System or equivalent
Pump	Agilent 1100 G1311A, Germany
Auto sampler	Agilent 1100 G1367B, Germany
Mass spectrometer	Applied Biosystems MDS SCIEX, API 3200 LC-MS/MS, (Toronto, Canada)
Mode of detection	Electron spray ionization (ESI).
Degasser	Agilent 1100 series
Analytical column	Waters, X-Bridge, C18, 4.6×50 mm, 5µm
Data analysis software	Analyst Software, Version 1.4.2
Pipettes	3-30, 30-300 and 300-1000 µL (Finnpipette, Thermo Electron Corporation, USA)
Eppendorfs	Tarsons (India)
Balance	Mettler Toledo AB 108
Cyclomixer	SPINIX (Tarson, India)
Centrifuge	Biofuge Heraeus, Germany
Filtration unit	Millipore, XI5522050 USA
Filtration membrane	0.45 µm, Millipore, Filter Type: HV, USA
Refrigerator	LG Ice Beam, 180 L, LG India Ltd, India
Deep Freezer	SANYO -80 °C (USA)
Ultra sonic cleaner	Branson, USA

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The LC-MS system used was an Agilent 1100 HPLC System, equipped with auto sampler, degasser, binary pump, thermostatic column compartment and mass selective detector fitted with Waters, X-Bridge, C18, (4.6×50 mm, 5µm) column. Electron spray ionization was -Mass spectrometry (ESI-MS) was kept in the positive mode and the operating parameters were: Ion spray voltage 5500 kV, drying gas temperature 500°C, Nebulizer gas pressure 35 psig, Auxillary gas pressure 40 psig, de-clustering potential for Linagliptin 71 kJ, for IS 65 kJ, Collision energy for Linagliptin 30 kJ, for IS 25 kJ, entrance potential is 8 kJ for both, exit potential for Linagliptin is 13 kJ, for IS 16 kJ. Analyte and IS were eluted as isocratic elution upto a total retention time of 1.45 min for Linagliptin and 1.20 min for IS, using mobile phase consisting of 0.1% formic acid: Acetonitrile (10:90 v/v) at a flow rate of 0.6 mL /min.

Preparation of primary and working stock solutions

Linagliptin primary stock solution: Accurately weighed 5.089 mg of Linagliptin (Purity 99.29%) which is equivalent to 5 mg was transferred into 5 mL clean and dry volumetric flask. After dissolving in 4 mL methanol, volume was made up to the mark using methanol to get 1.0 mg/mL primary stock solution. Further dilutions were done with methanol as shown in Table 2 and 3.

Preparation of primary stock (PS) and working stock (WS) solutions of Linagliptin:

Table 2:

Stock	Wt. (mg)	Purity (%)	Final vol.(mL)	Solvent	Conc. (µg/mL)
PS1	5.089	99.29	5	Methanol	1000

Table 3: Stocks

Stock	Conc. (ng/mL)
PS2	5000
WS1	2000
WS2	1000
WS3	500
WS4	200
WS5	100
WS6	50
WS7	20
WS8	10
WS9*	4000
WS10*	150
WS11*	30
WS12*	10

The PS and WS were stored at 2-8 °C
*Stocks for QC

Preparation of Calibration Standard (CS) Samples, Quality Control (QC) Samples and recovery standards (RS): Independently prepared stocks were used for the preparation of the calibration and QC samples. Calibration samples were readily prepared for every analytical run (Table 4). QC samples were prepared at four concentration levels.

Table 4:

Stock	Standard	Vol. spiked (µL)	Vol. Plasma (µL)	Conc. (ng/mL)
PS2	CS9	50	450	5000
WS1	CS8	50	450	2000
WS2	CS7	50	450	1000
WS3	CS6	50	450	500
WS4	CS5	50	450	200
WS5	CS4	50	450	100
WS6	CS3	50	450	50
WS7	CS2	50	450	20
WS8	CS1	50	450	10
WS9*	HQC	50	450	4000
WS10*	MQC	50	450	150
WS11*	LQC	50	450	30
WS12*	LLQC	50	450	10

PS and WS were stored at 2-8 °C, *Stocks for QC.

Calibration standards were prepared by addition of 50µL of stock solution into 450µL of the human plasma (10-5000ng/mL) and mixed well. Calibration standards of Linagliptin and IS were prepared by spiking the required amount of stock solutions into blank plasma obtained from healthy volunteers.

Processing of samples

An aliquot of 50µL of Stock spiked into 450µL of Human plasma and 50µL of IS was added then added 2mL of Ethyl acetate (EA), This was vortexed for 5min and centrifuged at 4000 rpm for 5min. Supernatant (1.6mL) was transferred and evaporate under nitrogen at 50°C and reconstituted with 500µL of mobile phase then added to vials 200µL and 10µL was injected into analytical column.

Preparation of Mobile Phase

Mobile Phase: Acetonitrile 900mL was taken into a solvent reservoir and added 100 mL of 0.1% Formic acid filtered through 0.45µm membrane. Mobile phase was sonicated approximately for 5 to 10 min and labeled.

Bioanalytical Conditions

Liquid Chromatography Conditions: **Table 5:**

Parameters and Conditions	
System	: Agilent 1100 HPLC Quaternary System
Mass spectrometer	: Applied Biosystems MDS SCIEX, API 3200 LC-MS/MS, (Toronto, Canada)
Mode of detection	: Electron Spray Ionization (ESI)
Analytical column	: Waters, X-Bridge, C18, (4.6×50 mm), 5µm
Mobile phase	: 0.1% formic acid: Acetonitrile (10:90 v/v)
Flow rate	: 0.6 mL/min
Retention time	: Linagliptin ~: 1.45 min
	Telmisartan ~: 1.20 min
Run time	: 3.0 min
Software version	: Analyst Software version 1.4.2
Auto Sampler Parameters	
Auto sampler rack	: 2 well plate (54 vials per plate)
Vol. of injection	: 10 µL
Temperature	: 4°C
Draw speed	: 200 µL/min
Eject speed	: 200 µL/min
Wash flush port	: 6 sec with acetonitrile

Validation Procedures

Selectivity and specificity: Human plasma was collected from six different healthy volunteers and processed according to the procedure of plasma processing and analysed to determine whether any interference peak is observed in the retention time of Linagliptin and IS peaks. The analyte response at the LLOQ should be at least 5 times the response compared to the blank plasma.

Response functions: Plasma CS samples (CS1-CS8) were prepared and analyzed on four different occasions according to the procedure, across the calibration range of 10 to 5000 ng/mL.

Accuracy and precision: The accuracy and precision of the analytical method was determined by analysing spiked QC samples, with the analyte at four different concentrations viz., at LLOQ, low, medium and high concentration range of the calibration curve. Six replicates at each QC levels were analysed on four different occasions and intra/inter day accuracy and precision were calculated against the corresponding nominal concentration [9, 10].

Matrix effect: The Matrix effect for this method examined by using concentrations equivalent to LLOQ. Analyze the samples with readily spiked calibration curve standards and two sets of quality control samples were prepared in already screened biological matrix.

$$\text{Analyte \% matrix effect} = \frac{\text{Mean of areas of analyte set 2}}{\text{Mean of areas of analyte set 3}} \times 100$$

$$\text{IS \% matrix effect} = \frac{\text{Mean of areas of IS set 2}}{\text{Mean of areas of IS set 3}} \times 100$$

Recovery: The % mean recovery of Linagliptin determined by comparing the mean peak area of six replicates of extracted plasma quality control samples against un-extracted quality control samples at low, medium and high concentrations. The internal standard recovery was determined by comparing the mean peak area of the internal standard in the extracted plasma QC samples at set 1 & 2 [11, 12].

$$\text{Analyte \% recovery} = \frac{\text{Mean of areas of analyte from set 1}}{\text{Mean of areas of analyte from set 2}} \times 100$$

$$\text{IS \% recovery} = \frac{\text{Mean of areas of IS from set 1}}{\text{Mean of areas of IS from set 2}} \times 100$$

Stability:

Stability in solution - Bench top: The bench top stability of Linagliptin in solution was investigated at ambient room temperature (21 ± 2°C) for 8 h at LQC and HQC concentration levels using four replicates and was compared with the 0 h.

Stability in Human plasma - Freeze/ Thaw cycle: The freeze/thaw stability in Human plasma was investigated for three freeze thaw cycles. Samples were thawed unassisted at room temperature and transferred back to the freezer (-80 ± 10°C) for a period minimum of 12 h before next F/T cycle. The cycle of thawing and freezing was repeated for two more times, and analysed on the third cycle. Samples were analysed at two concentrations using six replicates.

Stability in Human plasma – Bench top: The bench top stability of Linagliptin in Human plasma was investigated at ambient room temperature (21 ± 2°C) for 6 h at LQC and HQC concentration levels using six replicates.

Stability in the Auto sampler: The auto sampler stability of Linagliptin in the final processed samples, waiting for injection, was investigated at 4°C for approximately 24 h at four concentrations. Six replicates were injected after 24 h and compared with 0 h of previous day run.

Stability in Human plasma - Long-term: The long term stability in Human plasma at -80 ± 10°C is being investigated for 15 days at two concentration levels (at low and high concentration range of the calibration curve). Six replicates of each LQC and HQC samples were used for assessing the experiment of bench top stability [13, 14].

RESULTS

Accuracy and precision (Intra and inter- day Accuracy and Precision):

The assay was performed and the data for the determination of Linagliptin was presented. The intra

and inter-day assay accuracy was within 80 - 120 % of nominal concentration at LLOQ and 85 - 115 % at remaining test levels. Precision was within ± 20 % of mean value at LLOQ and ± 15 % at remaining test levels. Overall mean accuracy was more than 99 %.

Selectivity and specificity

Chromatograms of Human plasma did not show any interfering molecular ion peaks at the retention times of Linagliptin and IS. The analyte response at the LLOQ is 5 times the response compound to the blank plasma. Further this was substantiated with accuracy and precision in the measurement of concentration at LLOQ (10ng/mL).

Response functions

For the determination of Linagliptin, the linear regression of the peak area ratios of drug to IS versus *the* concentration (range: 10-5000ng/mL) was plotted and 1/X*X weighting was applied. Analyst software, version 1.4.2 was used; correlation coefficient (r) ≥ 0.99 or better was obtained.

For every calibration curve the calibration concentrations were back calculated from the response. The % accuracy from expected concentration at all test levels were between 93.40 and 104.13%, proving linearity. The CV % values were in the range of 5.04 to 13.44 %.

Recovery

A better recovery should be more than 90%. The overall recovery of Linagliptin and IS was 92.5 and 89.9 %, respectively.

Matrix effect

Analyzed the samples with readily spiked calibration curve standards and two sets of quality control samples were prepared in already screened biological matrix. The matrix effect of Linagliptin and IS was 5.51 and 1.33 %, respectively.

Stability:

Stability in solution- Bench top: The bench top stability of stock solution of Linagliptin was in comparison with the freshly prepared stock at low, medium and high concentration and the accuracy was between 98.33 to 100.55%.

Stability in Human plasma – Freeze/thaw: The compound remaining after three freeze thaw cycles was found to be in range of 101.12 to 102.23 percent of samples which has not undergone any F/T cycle.

Stability in Human plasma – Bench top: There was no evidence of degradation of Linagliptin in the plasma after 6 h at room temperature (21 ± 2°C). Percent remaining after incubation for 6 h at bench top was in range of 102.70 to 102.0 of samples analyzed just after spiking into plasma.

Stability in the auto sampler: There was no evidence of degradation of Linagliptin in the auto sampler for approximately 24 h at 4°C. Percent of compound remaining after 24 h incubation in auto sampler was found in the range of 94.02 to 97.64 of concentration at 0 h

Stability in Human plasma - long term: Long term stability for a period of 25 days at -80 ± 10°C was established and compared with day 1 data. Percent remaining after 25 days at -80 ± 10°C was in range of 98.31 to 99.79 %.

Table 6:

	Plasma bench top stability after 6 h. <-------- Concentration (ng / mL.) -------->			
	LQC		HQC	
	0 h	6 h	0 h	6 h
	28.9	32.100	4001	4023.0
	28.5	29.800	4023	4012.0
	31.2	31.200	3969	4020.0
	28.9	30.200	3817	4121.0
	31.0	30.000	4010	4210.0
	30.1	30.400	4002	3892.0
n(numb. of samples)	6	6	6	6
C_{mean}	29.8	30.58	3970	4046
S.D.	1.20	0.84	77.21	108.07
% C.V.	4.04	2.75	1.94	2.67
C_{exp}	30.00	30.00	4000.00	4000.00
%ACC	99	102	99	101.199
%ACC (comp. 0 h)		102. 69		102.00

Table 7: 25 Days long term stability in Plasma matrix at -80°c

	<-------- Concentration (ng/mL.) -------->			
	LQC		HQC	
	Day 1	Day 25	Day 1	Day 25
	28.0	29.800	3930	4020.0
	30.4	30.2000	4116	4026.0
	32.1	30.200	3989	4003.0
	30.0	30.200	4023	4060.0
	32.5	31.200	3983	3895.0
	30.2	29.600	3982	3963.0
N (numb. of samples)	6	6	6	6
C_{mean}	30.53	30.05	4004	3995
S.D.	1.60	0.76	64.89	58.13
% C.V.	5.22	2.54	1.62	1.46
C_{exp}	30.00	30.00	4000.00	4000.00
%ACC	101.79	100.01	100.1	99.9
%ACC (comp. 0 h)		98.31		99.79

Table 8: Calibration concentrations back-calculated from the coefficients Concentration (ng/mL)

Cexp	Run-1	Run-2	Run-3	Mean	S.D	CV (%)	% Acc
10	9.8	9.6	10.5	10.0	0.5	4.7	99.7
20	19.5	19.8	18.6	19.3	0.6	3.2	96.5
50	48.0	49.0	52.0	49.7	2.1	4.2	99.3
100	86.2	89.6	108.0	94.6	11.7	12.4	94.6
200	189.2	198.2	208.0	198.5	9.4	4.7	99.2
500	499.8	503.2	496.2	499.7	3.5	0.7	99.9
1000	986.2	999.2	1036.0	1007.1	25.8	2.6	100.7
2000	1989.3	2065.0	2015.0	2023.1	38.5	1.9	101.2
5000	4963.0	5036.0	5075.0	5024.7	56.9	1.1	100.5

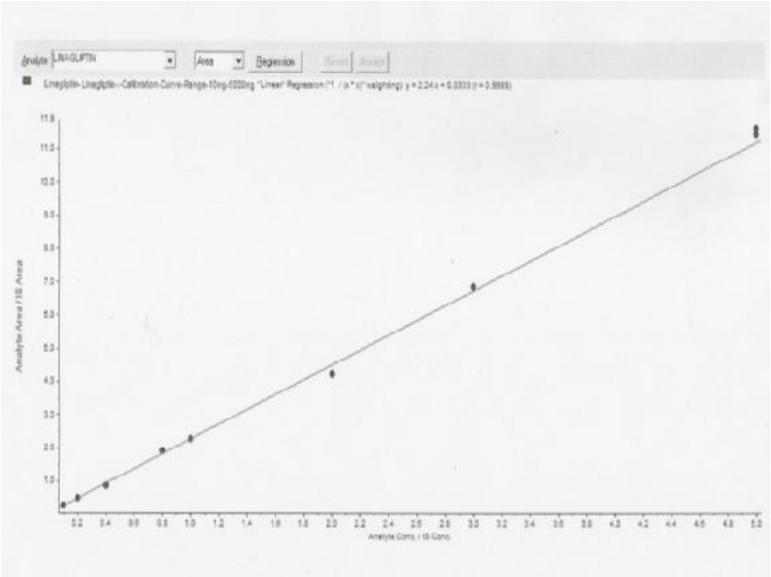

Figure 2: Calibration curve of Linagliptin

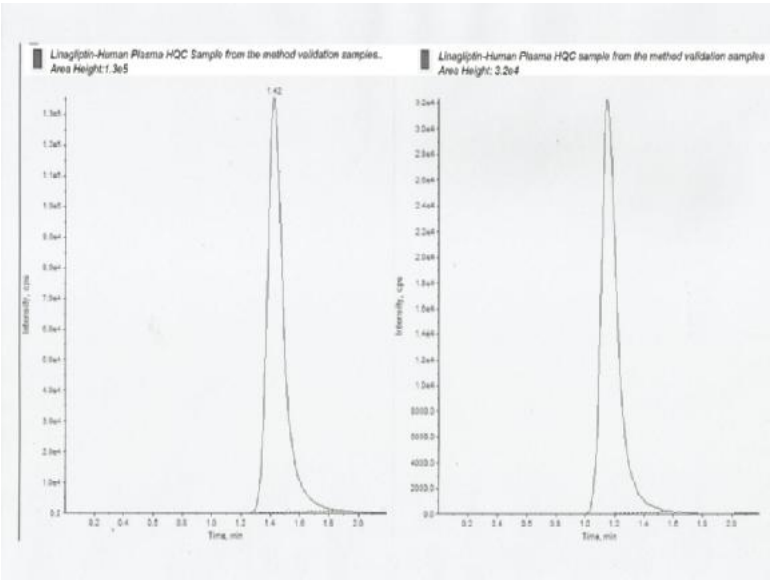

Figure 3 & 4: Linagliptin-MRM-Linagliptin-HQC-4000ng sample peak Linagliptin-MRM-Human-plasma. (Left side fig.): Area: 1.60e+006counts Height: 1.35e+005cps RT: 1.42 min (Right-side fig.): Area: 2.52e+005 counts Height 3.23e+004 cps.

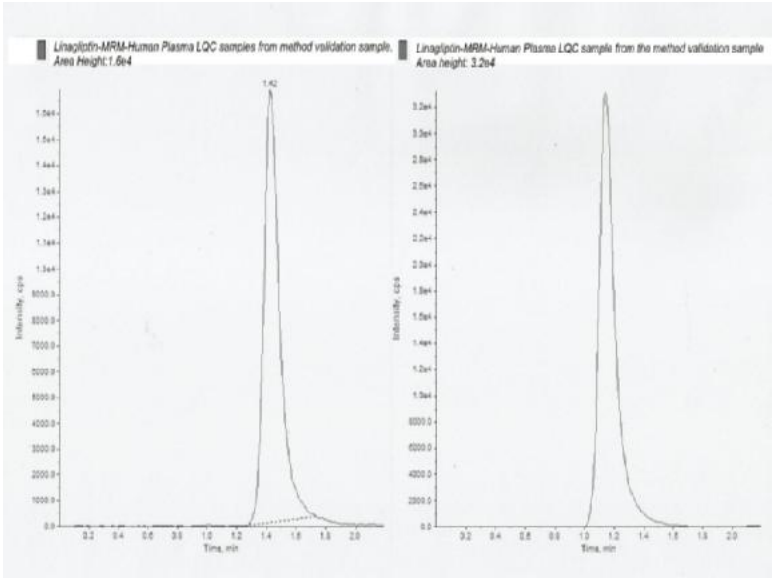

Figure: 5 & 6: Linagliptin-MRM-Linagliptin-LQC-30ng sample peak Linagliptin-MRM-Human-plasma. (Left side fig.): Area: 1.260e+005counts Height: 1.68e+004cps RT: 1.42 min (Right-side fig.): Area: 2.60e+005 counts Height 3.33e+004 cps.

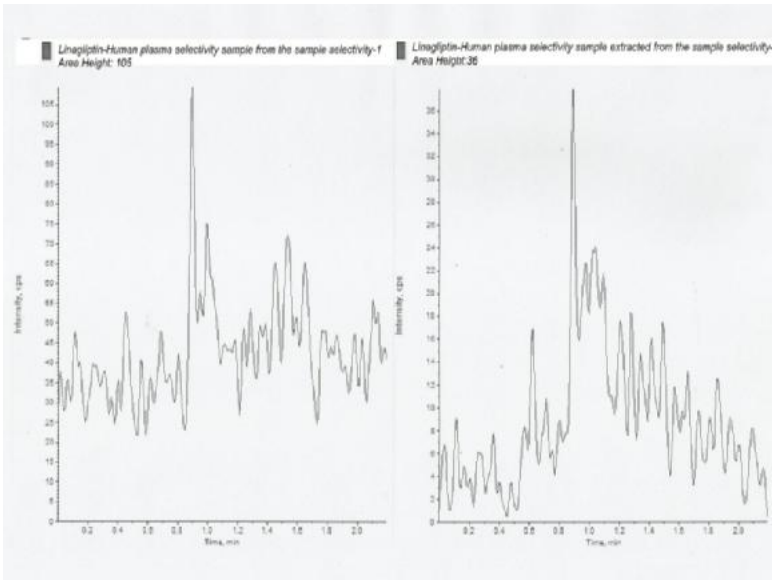

Figure 7 & 8: Selectivity of Linagliptin

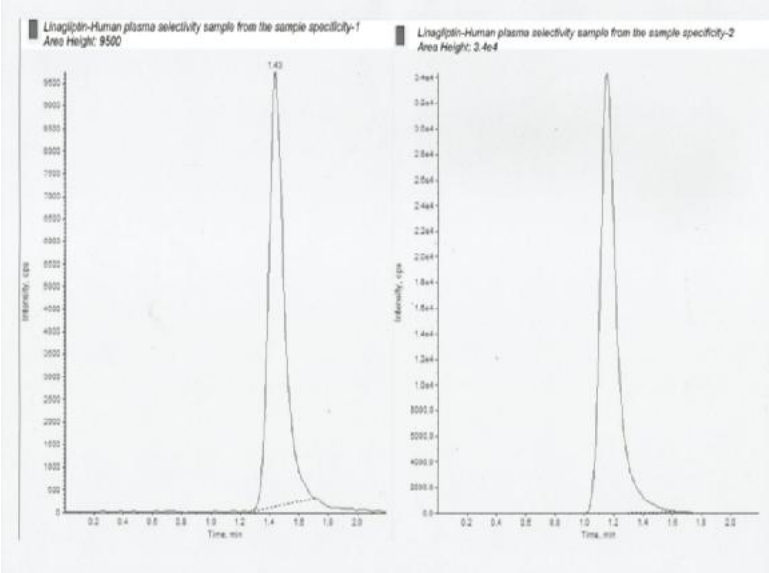

Figure 9&10: Linagliptin-MRM-Linagliptin-LLOQ-Specificity sample peak- Linagliptin-MRM-Human-plasma. (Left side fig.): Area: 7.07e+004 counts Height: 9.65e+003cps RT: 1.43 min (Rightside fig.): Area: 2.67e+005 counts Height 3.44e+004 cps.

CONCLUSION

The proposed method of LC-MS/MS has proved to be simple, sensitive, accurate, precise and reliable. The method is specific due to the selectivity of the mass spectrometry. So the proposed study is proved to apply this method for the estimation of Linagliptin in human plasma.

ACKNOWLEDGEMENT

We gratefully acknowledge our dynamic principal Dr. D. Satyavathi, (Sree Dattha Institute of Pharmacy) for her constant encouragement, support and valuable suggestions throughout the project work.

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Source of support: Arifa Begum, Professor, Sree Dattha Institutte of Pharmacy.
Conflict of interest: None Declared