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Original Research Paper

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF LINAGLIPTIN AND METFORMIN DRUGS IN HUMAN PLASMA BY RP-HPLC METHOD

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ABSTRACT

A simple, rapid, precise and accurate high performance liquid chromatography method was developed for simultaneous determination of linagliptin and metformin in human plasma. The analytes were extracted by protein precipitation technique and chromatograph using a mobile phase consisting of acetonitrile and 0.01M di-potassium hydrogen phosphate buffer in ratio of 75:25 and adjusting pH 7.0 with orthophosphoric acid using Grace vyadyec genesis CN (150 × 4.6 mm, 4 µm) column. The flow rate 1.0 mL min^{-1} and UV detection at 237 nm was employed. The retention time for linagliptin and metformin and internal standard (phenformin) was 4.95, 15.41 min and 11.06 min respectively. Linearity for linagliptin and metformin was found to be in the range of 1-32 ng/mL for both drugs respectively. The method was validated as per the USFDA guidelines and the results were within the acceptance criteria for selectivity, sensitivity, linearity, precision, accuracy, recovery stability of solution, stability of solution in plasma and dilution integrity.

Keywords: Linagliptin, Metformin, Phenformin, Protein precipitation, Human plasma, RP-HPLC, Simultaneous determination.

INTRODUCTION

The combination of linagliptin and metformin is available as tablets formulation for oral use in diabetes. metformin (1-carbamimidamido-N,N-Dimethylmethanimidamide is biguanides introduced in 1950 as glucose-lowering agents to treat non-insulin-dependent diabetes mellitus (NIDDM).It reduces elevated blood glucose concentration in diabetic patients, but it does not increase insulin secretion. Biguanide is used alone or in combination with insulin or chlorpropamide. It is reported in pharmacopoeias such as BP1 and USP2. Linagliptin (8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1[(4methylquina-zolin -2-yl) methyl]-2, 3, 6, 7-tetrahydro-1H-purine-2, 6-Dione) linagliptin is not extensively metabolized, 90% of dose is excreted unchanged. The small portion of drug that is metabolized, the main metabolite is CD 1790 and is pharmacologically inactive. It is not reported in pharmacopoeias such as BP, USP and IP.[1-7] Several HPLC methods are reported in combination with other drugs for the determination of metformin in plasma, the literature for its analysis. However, no method is reported for simultaneous determination of linagliptin and metformin in human plasma by RP-HPLC in any literature. In the present investigation, a specific RP-HPLC method is described for the simultaneous determination of linagliptin and metformin drugs with human plasma.[8-12]

MATERIAL AND METHODS

Instrumentation

The HPLC system used was HPLC Shimadzu LC-2010C HT, series equipped with a 0.1 to 100 µL sample loop, and LC-100 UV Detector. The output signal was monitored and integrated using Lab Solution version software. Grace vyadyec genesis CN (150 × 4.6 mm, 4 µm) column was used for the separation.

Materials

The drug sample of linagliptin obtained from Manus Aktteva Bio Pharma, Ahmedabad and metformin was obtained from Intas Pharmaceuticals Ltd, Ahmedabad and phenformin was obtained from Cadila Pharmaceuticals Ltd, Ahmedabad. Acetonitrile HPLC Grade (Fisher Scientific, India), HPLC Grade water (Fisher Scientific, India), HPLC Grade methanol, dichloromethane (DCM), diethyl ether (DEE), tertiary butyl methyl ether (TBME), (ethyl acetate)EA, trichloro acetic acid (TCA), and perchloric acid (PCA) from (Fisher Scientific, India) are used in the study.

Chromatographic Conditions

The analysis was carried out on HPLC Shimadzu LC-2010C HT system using a Grace vyadyec genesis CN (150 × 4.6 mm, 4 µm) column as a stationary phase with UV detection at 237 nm at ambient room temperatures using a 10 µL injection volume.

Mobile Phase

A mixture of acetonitrile and 0.01M di-potassium hydrogen phosphate buffer in ratio of (75:25) and adjusted to pH 7.0 using o-phosphoric acid, filtered, degassed and used. 0.01M Di-potassium hydrogen phosphate buffer (pH 7.0) prepared in 100 ml volumetric flask, add 17.41 gm of di-potassium hydrogen phosphate and dissolve it in some of amount of HPLC grade water, and make up to volume with HPLC grade water. Adjust the pH 7.0 of resultant buffer by orthophosphoric acid, as required.[13-16]

Preparation of Solution

Stock solution of linagliptin was prepared by, linagliptin 5 mg is accurately weighed on analytical precision balance and transferred in 50 ml of volumetric flask and dissolve in some amount of HPLC grade methanol, shake it until it dissolve and than make up to mark with HPLC grade methanol which was labeled as stock-1 solution (100 µg/ml). From that stock-1 solution, 1 ml was transferred by means of pipette in 10 ml of volumetric flask which was than make up to mark with HPLC grade methanol which was finally labelled as Stock-2 solution (10 µg/ml). Stock solution of metformin was prepared by, metformin 10 mg was accurately weighed on analytical precision balance and transferred in 50 ml of volumetric flask and dissolve in some amount of HPLC grade methanol, shake it until it dissolve and than make up to mark with HPLC grade methanol which was labelled as Stock-1 solution (200 µg/ml). From that Stock-1 solution, 0.5 ml was transferred by means of pipette in 10 ml of volumetric flask which was than make up to mark with HPLC grade methanol which was finally labelled as Stock-2 solution (10 µg/ml).

Working standard solution-1 (WS-1) of was prepared by, using a calibrated micropipette, 100 µl of each LNG and MET Stock-2 solutions were added to 10 ml volumetric flask and volume made up to 10 ml with methanol which have 100 ng/ml of LNG and MET respectively.

Working standard solution-2 (WS-2) of was prepared by using a calibrated pipette, 1 ml of WS-1 was added to 10 ml volumetric flask and volume made up to 10 ml with methanol which have 10 ng/ml of LNG and MET respectively. All the solutions were stored in a refrigerator at 2-8 ^{o}C until use

Sample Preparation

Protein precipitation with acid: Drug+ 200µl spiked plasma + 50 µl IS + 50 µl of 2% perchloric acid + 1000 µl acetonitrile and vortex to mix. Centrifuged for 07-10 minutes at 8000 rpm at 4ºC and supernatant was collected. Supernatant was evaporated to dryness using nitrogen gas and reconstituted with 50 µl of mobile phase, and 10µl sample was analysed.

Preparation of Plasma Calibration Curve Standards and Quality Control Standards

To prepare calibration curve standards and quality

control standards, take volume as mentioned in table, evaporate solvent using nitrogen evaporator.[17] Add 200 µl Human plasma which had been checked for specificity and vortex for 30 sec. then follow sample preparation method:

Details	Vol. pipette from	Vol. pipette (µl)	Concentration (ng/ml) Linagliptin	Concentration (ng/ml) Metformin
S1	WS-1	320	32	32
S2	WS-1	160	16	16
S3	WS-1	100	10	10
S4	WS-1	80	8	8
S5	WS-2	400	4	4
S6	WS-2	200	2	2
S7	WS-2	100	1	1
LLOQ	WS-2	100	1	1
HQC	WS-1	200	20	20
MQC	WS-1	90	9	9
LQC	WS-2	300	3	3

Method Development

The mobile phase consisting of acetonitrile and 0.01M Di-potassium hydrogen phosphate buffer in varying proportions and change in pH was tried and finally ratio of 75:25 (pH-7.0 adjusted with orthophosphoric acid) was selected because it was found to give good separation for the peaks of linagliptin (R_t-5.55 min) and metformin (R_t-7.48 min) and IS (R_t-7.48 min) respectively as shown in the figure 1. In addition to this, UV spectra of individual drugs were recorded at the wavelength range from 200 to 400 nm and the response for optimization was compared. The choice of wavelength 237 nm was considered satisfactory, permitting the detection of drugs with adequate sensitivity.

Method Validation

The method was validated in accordance with USFDA guidelines and EMEA guidelines.[18-20]

System Suitability

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture equivalent to MQC (Mid quality control sample) concentration of the calibration curve for all analytes and 1000 ng/ml for IS. System suitability was performed at the start of the method validation and on each day as a first experiment.

Selectivity

The selectivity of HPLC Method was established by screening the standards blanks of different lots of Human Plasma. Six different lots of plasma were screened for the Experiment. All six lots were found to be free of Significant interferences at the Retention time of all analytes in standard blank samples was ≤ 20.00% of the area of the drug in the Extracted LLOQ (Lower Limit of Quantification) Samples; area of peak at the Retention time of IS in the standard blank samples was ≤ 5.00% of the area of the IS in the Extracted LLOQ Sample as per acceptance limit.

Sensitivity

The sensitivity of the method was evaluated by analyzing 6 LLOQ at 1ng/ml for LNG and MET respectively.

Calibration Curve/Linearity

The linearity of the method was determined by using a regression analysis of standard plots associated with a seven-point standard curve. All the three calibration curves analyzed during the course of validation were found to be linear for the

standard concentration ranging from 1-32 ng/ml range for LNG and MET.

Precision

The precision of the HPLC-UV method was evaluated by the %CV at different concentration levels corresponding to LLOQ, LQC, MQC and HQC during the course of validation.

Within-batch precision

The %CV of back calculated concentrations for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels with four replicates for LNG and MET were spiked combined with plasma sample and were being analyzed by HPLC.

Between-batch precision

The %CV of back calculated concentrations for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels from three different batches of four replicates at each QC levels for LNG and MET were spiked combined with plasma sample and were being analyzed by HPLC.

Accuracy

The accuracy of the HPLC-UV method was evaluated by the % nominal concentration at different concentration levels corresponding to LLOQ, LQC, MQC and HQC during the course of validation.

Within-batch accuracy

The percentage nominal of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC and HQC concentration levels with four replicates for LNG and MET were spiked combined with plasma sample and were being analyzed by HPLC.

Between-batch accuracy

The percentage nominal of back calculated concentrations for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels from three different batches of four replicates at each QC levels for LNG and MET were spiked combined with plasma sample and were being analyzed by HPLC.

Recovery

The percentage mean recoveries were determined by measuring the responses of the quality control samples spiked into plasma against respective aqueous quality control samples at LQC, MQC and HQC levels. Three samples at each level were analyzed after extraction of each individual drug in separate solvent and % Nominal concentration of the sample was calculated.

Short Term Stock Solutions Stability of Analytes and Internal Standard

Short term stock solution stability for the LNG, MET and IS at concentration 100 µg/ml were determined by using stock solution dilution equivalent to concentration of 1000 ng/ml for LNG, MET and IS respectively, after storage of stock solution over a period of 6 hours at room temperature. Stability was assessed by comparing against the freshly prepared stock. The % mean stability was calculated.

Long Term Stock Solutions Stability of Analytes and Internal Standard

Long term stock solution stability for the LNG, MET and IS at concentration 100 µg/ml were determined by using stock solution dilution equivalent to concentration of 1000 ng/ml for LNG, MET and IS respectively, after storage of primary stock solution over a period of 20 days at 2-8°C. Stability was assessed by comparing against the freshly prepared stock. The % mean stability was calculated.

Bench Top Stability

Bench top stability of the spiked quality control samples was determined for a period of 6 hr. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards.

Auto Sampler Stability

Auto sampler stability of the processed quality control samples was determined for a period of 24 hours by storing them in auto sampler maintained at 15°C. Stability was assessed by comparing processed sample against the freshly spiked calibration standards

Freeze Thaw Stability

Freeze thaw stability of the spiked quality control samples was determined after three freeze thaw cycles stored at -80 °C. Stability was assessed by comparing them against the freshly spiked

calibration standards.

Long Term Stability

Long term stability of the spiked quality control samples was determined after stored at -80 °C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards.

Dilution Integrity

The dilution integrity of the method was evaluated by diluting the stock concentration sample as spiked standard at concentration 1000 ng/ml for LNG & MET, 1000 ng/ml conc. samples were diluted to 500 ng/ml (2 times) and 1000 ng/ml samples were diluted to 250 ng/ml (4 times) in blank plasma. The precision and accuracy for dilution integrity standards at 1/2 and 1/4 dilution were determined by analyzing the samples against calibration curve standards

RESULTS AND DISCUSSION

System Suitability

The %CV of the retention times was found to be ≤ 1.42 for all analytes and IS. The %CV of the peak area was found to be ≤ 3.7 for all analytes and IS. Acceptance limit for retention time (Rt) deviation and area deviation 2% and 5%CV respectively were passed. The results are summarized in Table-1.

Selectivity

All six lots were found to be free of Significant interferences at the Retention time of all analytes in standard blank samples was ≤ 20.00% of the area of the drug in the Extracted LLOQ (Lower Limit of Quantification) Samples; area of peak at the Retention time of IS in the standard blank samples was ≤ 5.00% of the area of the IS in the Extracted LLOQ Sample as per acceptance limit. In optimization trials we choose such method where plasma lots were found to be free of significant interferences at the Retention time of all analytes in standard blank samples The Result is summarized in Table-2.

Sensitivity

The precision and accuracy for MET at LLOQ level were found to be 4.81 %CV and 96 to 106% nominal respectively. Acceptance criteria is at least 67% of the sample should be within 80-120% of nominal and precision should be <20 %CV. The results are summarized in the Table-3.

Calibration Curve/Linearity

Representative calibration curve is shown in figures which are obtained during the precision and accuracy batch. The average correlation coefficient (R^2) was ≥ 0.99 during the course of validation. Data of calculated calibration standard concentration are shown in Table-4 and Table-5 respectively

Precision

Within batch precision

The %CV of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC and HQC concentration levels with four replicates for LNG and MET were within 1.325 to 9.823% and 0.248 to 3.382% respectively. Acceptances criteria are that at least 67% of QC samples must be within 15% except LLOQ where limit is within 20%.

Between batch precision

The %CV of back calculated concentrations for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels from three different batches of four replicate at each QC levels were found within 1.632 to 7.708% and 0.784 to 2.883% for LNG and MET respectively. Acceptances criteria are that at least 67% of QC samples must be within 15% except LLOQ where limit is within 20%. The results are shown in Table-7,8 and 9 summarized in the Table-6.

Accuracy

Within batch accuracy

The percentage nominal of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC and HQC concentration levels with four replicates for LNG and MET respectively were within 97-103% and 99-104% respectively. Acceptance criteria are that at least 67% of QC samples must be within 85-115%.

Between batch accuracy

The percentage nominal of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC and HQC concentration levels with four replicates of three different batches for all LNG and MET were within 85-102% and 99-

102% respectively. Acceptances criteria are that at least 67% of QC samples must be within 85-115%. The results are shown in Table-7,8 and 9 summarized in the Table-6.

Recovery

The % mean recovery of drugs acceptable limit was % CV of 15 and that of IS was % CV of 20.The results are shown in Table-10 and 11.

Short Term Stock Solution Stability of Analytes and Internal Standard

Short term stock solution stability for the LNG, MET and IS at concentration 100µg/ml were determined by using stock solution dilution equivalent to concentration of 1000ng/ml for LNG, MET and IS respectively, after storage of stock solution over a period of 6 hours at room temperature. Stability was assessed by comparing against the freshly prepared stock. The % mean stability was found to be 96.88, 97.07 and 97.88% for LNG, MET, and IS respectively which is within the acceptance limit of 90.00 – 110.00%. The results are summarized in the Table-12.

Long Term Stock Solution Stability of Analytes and Internal Standard

Long term stock solution stability for the LNG, MET and IS at concentration 100µg/ml were determined by using stock solution dilution equivalent to concentration of 1000ng/ml for LNG, MET and IS respectively, after storage of primary stock solution over a period of 20 days at 2-8°C. Stability was assessed by comparing against the freshly prepared stock. The % mean stability was found to be 91.68, 90.14, and 95.87% for LNG, MET, and IS respectively which is within the acceptance limit of 90.00 – 110.00%. The results are summarized in the Table-12.

Bench Top Stability

Bench top stability of the spiked quality control samples was determined for a period of 6 hr. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 96.14% & 95.72% and 98.13% & 97.69% for LNG and MET respectively. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should fail. Results are summarized in Table-13 and 14 for LNG and MET respectively.

Auto Sampler Stability

Auto sampler stability of the processed quality control samples was determined for a period of 24 hours by storing them in auto sampler maintained at 15°C. Stability was assessed by comparing processed sample against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 91.97% & 94.11% and 97.66% & 98.38% for LNG and MET respectively. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should fail. Results are summarized in Table-13 and 14 for LNG and MET respectively.

Freeze Thaw Stability

Freeze thaw stability of the spiked quality control samples was determined after three freeze thaw cycles stored at -80 °C. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 92.00% & 91.54% and 98.95% & 96.91% for LNG and MET respectively. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should fail. Results are summarized in Table-13 and 14 for LNG and MET respectively.

Long Term Stability

Long term stability of the spiked quality control samples was determined after stored at -80 °C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 92.02% & 89.13% and 97.11% & 99.46% for LNG and MET respectively. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should fail. Results are summarized in Table-13 and 14 for LNG and MET respectively.

Dilution Integrity

The dilution integrity of the method was evaluated by diluting the stock concentration sample as spiked standard at concentration 1000ng/ml for LNG & MET, 1000ng/ml conc. samples were diluted to 500ng/ml (2 times) in blank plasma and The precision and accuracy for dilution integrity standards at 1/2 dilution were determined by analyzing the samples against calibration curve standards. The precision for dilution integrity of 1/2 was found to be 4.94 and 6.57% for LNG and Met respectively which is within the acceptance limit of <15%. The % mean accuracy for dilution integrity of 1/2 was found to be within 93.46-107.48% and 93.68-110.02% for LNG and MET respectively which is within acceptance limit 85.00-115.00%. The results are summarized in Table-15.

CONCLUSION

A Simple, Rapid and Economic RP-HPLC method for simultaneous determination of Linagliptin and Metformin from human plasma was developed and validated. All the analytes and internal standard (Phenformin) were chromatographed on reverse phase CN column-grace vyadec genesis (150 mm × 4.6 mm × 4 µm) using Acetonitrile : di-potassium hydrogen phosphate buffer (0.01M, pH=7) 75:25 mobile phase at flow rate 1 ml/min over 18 min run time. Detection of analysis was performed at their specific wavelength by UV respectively. detector. Linagliptin and Metformin were extracted from human plasma using different solvents and analyzed by RP-HPLC method. Developed method was optimized prior to validation studies in terms of optimization of extraction procedure, mobile phase composition, flow rate, etc. The total chromatographic run time was 18 min with retention time for Linagliptin, Metformin and Internal Standard (Phenformin) as 4.95 min, 15.41 min and 11.06 min respectively. The developed method was validated in human plasma matrix, with a range of 1 to 32 ng/ml for Linagliptin and Metformin which is at very sensitive level even using simple mobile phase. The method was validated for all the parameter such as specificity, sensitivity, linearity, accuracy, precision, recovery, dilution integrity, stability as per USFDA and EMEA guidelines on bioanalytical method validation

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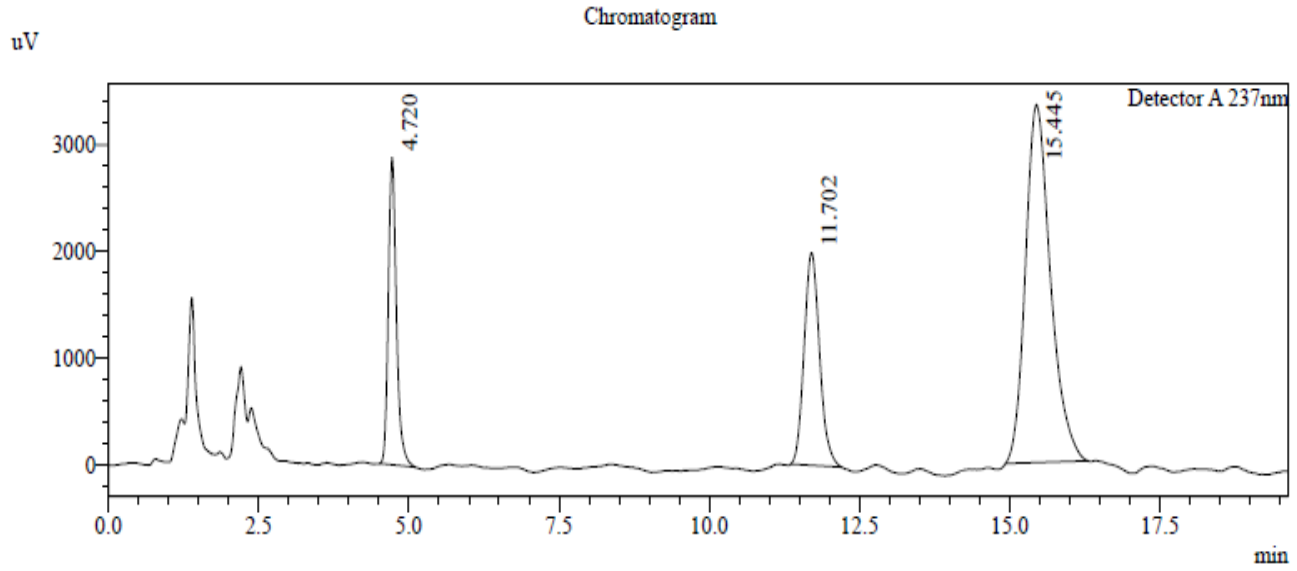

Figure1: Chromatogram of unextracted sample

Chromatogram of Unextracted sample, LNG(Rt-4.7min), PHEN(Rt-11.7min) and MET (Rt-15.4min)

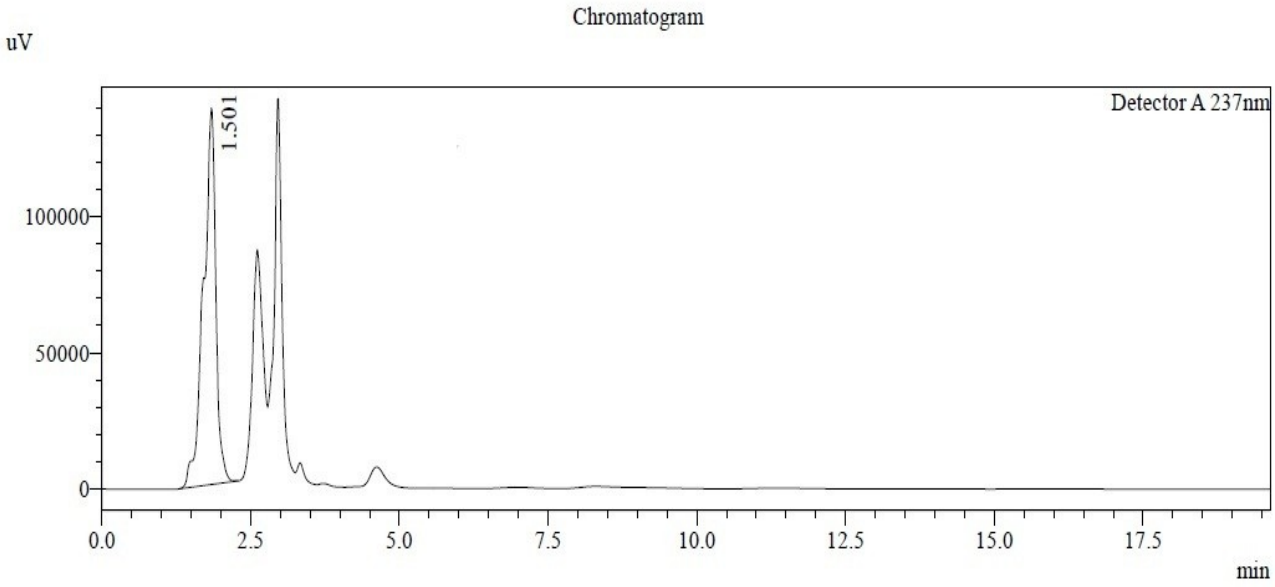

Figure 2: Chromatogram of blank plasma sample

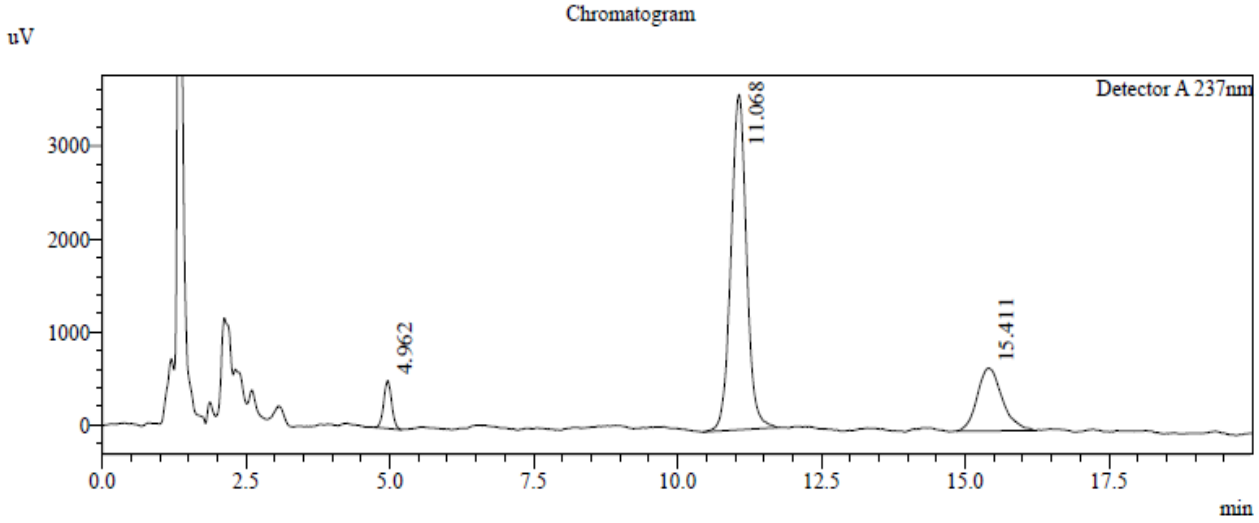

Figure 3: Chromatogram of extracted plasma sample

Chromatogram of Extracted sample, LNG (Rt-4.9min), PHEN (Rt-11min) and MET (15.4min)

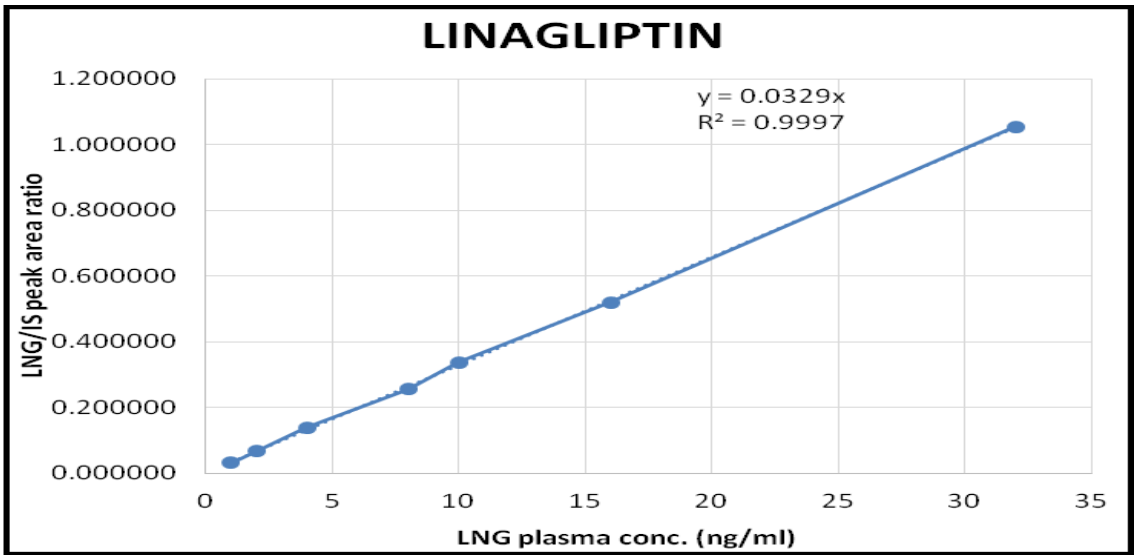

Figure 4: Linearity plot of Linagliptin

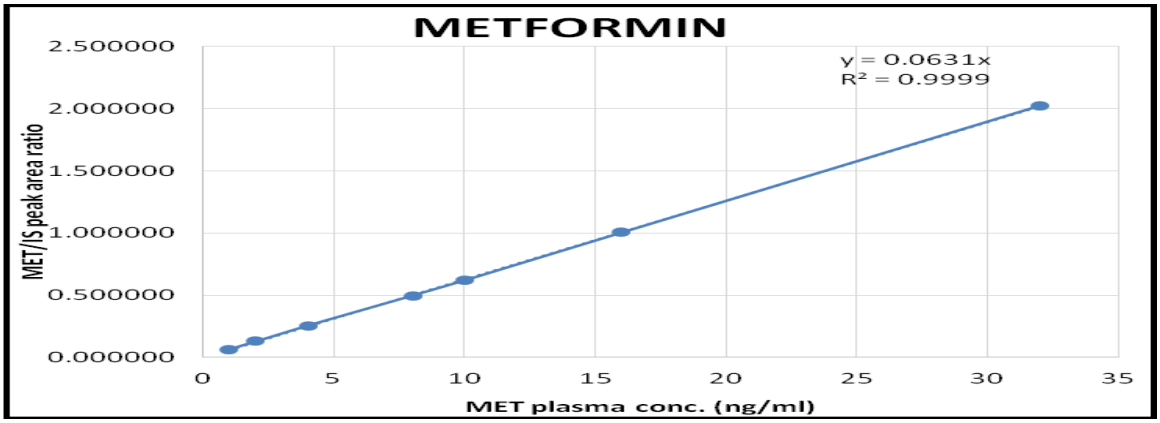

Figure 5: Linearity plot of Metformin

Table 1: System Suitability data

	LNG		MET		IS	
	Area	Rt (Min)	Area	Rt (Min)	Area	Rt (Min)
MQC Unex. sample	9439	4.77	18489	15.47	31601	11.71
	9314	4.77	19605	15.47	31863	11.69
	9603	4.76	19756	15.48	31765	11.68
	9498	4.73	18145	15.53	31651	11.69
	9402	4.74	19213	15.55	31872	11.68
	9122	4.73	18736	15.58	31132	11.70
	9407	4.79	19742	15.47	31407	11.53
	9179	4.79	18120	15.59	31279	11.57
	9246	4.80	18145	16.17	31246	11.65
Average	9356.6	4.764	18883.4	15.590	32060.5	11.655
SD	155.21	0.0265	705.20	0.2227	1108.85	0.0628
%CV	1.6588	0.5564	3.73	1.4289	3.45	0.5394

Table 2: Selectivity data

Plasma ID	Blank Area	LLOQ Area	% Interference
LINAGLIPTIN			
SDRP-1	N.A	961	N.A
SDRP-2	10	953	1.04
SDRP-3	N.A	932	N.A
SDRP-4	N.A	939	N.A
SDRP-5	N.A	929	N.A
SDRP-6	8	922	0.86
METFORMIN			
SDRP-1	N.A	1976	N.A
SDRP-2	N.A	1968	N.A
SDRP-3	N.A	2098	N.A
SDRP-4	N.A	2143	N.A
SDRP-5	14	1829	0.76
SDRP-6	N.A	1847	N.A

Table 3: Sensitivity data

Sample/Parameter	LNG		MET	
	Cal Conc. (ng/ml).	% of Nominal Conc.	Cal Conc. (ng/ml)	% of Nominal Conc.
LLOQ-1	0.978	98	0.962	96
LLOQ-2	0.952	95	0.940	94
LLOQ-3	1.022	102	0.967	97
LLOQ-4	1.040	104	0.987	99
LLOQ-5	1.032	103	1.049	105
LLOQ-6	1.021	102	1.055	106
Nominal Conc. (ng/ml)	1		1	
Mean Cal. Conc. (ng/ml)	1.0075		0.9933	
SD	0.0346		0.0478	
% CV	3.43		4.81	

Table 4: Calibration curve (Linearity) data of Linagliptin

Nominal Conc.(ng/ml)	Back calculated concentrations (ng/ml)			Average	%CV
	1	2	3		
1	1.0089	0.9819	1.0206	1.00	1.97
2	2.1849	2.0953	2.1878	2.16	2.43
4	3.9828	4.3525	3.8776	4.07	6.12
8	8.5371	7.9852	8.0253	8.18	3.76
10	10.6170	10.5297	10.2210	10.46	1.98
16	16.6976	16.2477	15.9596	16.30	2.28
32	32.2265	32.9610	33.1235	32.77	1.45
Slope	0.032	0.032	0.037	0.033	8.57
R^2 value	0.999	0.999	0.998	0.998	0.05

Table 5: Calibration curve (Linearity) data of Metformin

Nominal Conc.(ng/ml)	Back calculated concentrations (ng/ml)			Average	%CV
	1	2	3		
1	0.9763	0.9928	1.0322	1.00	2.87
2	1.9337	1.8980	2.1066	1.98	5.63
4	4.1133	4.2912	4.0690	4.16	2.82
8	8.1095	8.1453	7.8916	8.05	1.70
10	10.2155	10.4819	9.8483	10.18	3.12
16	16.2636	16.4130	15.9574	16.21	1.43
32	31.7596	31.7782	32.1201	31.89	0.63
Slope	0.068	0.075	0.063	0.068	8.77
R^2 value	0.999	0.999	0.999	0.999	0.00

Table 6: Summary of precision and accuracy data

Sample ID	Precision (% CV)		Accuracy (% of nominal Conc.)	
	Linagliptin	Metformin	Linagliptin	Metformin
[a] Intra-day (n = 4)				
LLOQ	2.715	3.382	104	102
LQC	9.823	3.341	97	99
MQC	1.983	1.944	103	102
HQC	1.325	0.248	103	104
[b] Inter-day (n = 12)				
LLOQ	1.632	2.883	102	99
LQC	7.708	2.398	85	99
MQC	2.391	1.613	89	101
HQC	2.088	0.784	94	102

Table 7: Precision and Accuracy batch no-1

Conc. (ng/ml)	LINAGLIPTIN				METFORMIN			
	LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC
Nominal concentration	1	3	9	20	1	3	9	20
Estimated concentration	1.009	1.893	5.458	15.882	0.976	2.928	8.788	19.68
	0.982	1.874	5.461	15.478	0.954	2.855	8.712	19.40
	0.992	1.884	5.824	14.414	0.992	2.913	8.940	19.93
	1.009	2.056	5.774	15.531	0.917	2.871	8.794	19.78
Average	0.998	1.927	5.629	15.326	0.960	2.892	8.808	19.70
SD	0.013	0.087	0.197	0.634	0.033	0.034	0.096	0.221
%CV	1.340	4.491	3.501	4.138	3.390	1.186	1.085	1.120
%Nominal average	100	64	63	79	96	96	98	99

Table 8: Precision and Accuracy batch no-2

Conc. (ng/ml)	LINAGLIPTIN				METFORMIN			
	LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC
Nominal concentration	1	3	9	20	1	3	9	20
Estimated concentration	1.022	3.090	9.079	20.333	1.030	3.091	9.341	20.789
	1.022	2.652	8.911	20.178	0.993	2.925	9.289	20.304
	1.040	3.027	9.082	20.092	1.013	2.951	9.377	20.514
	1.029	2.605	9.286	20.457	0.989	3.057	9.009	20.457
Average	1.028	2.843	9.089	20.265	1.006	3.006	9.254	20.516
SD	0.009	0.251	0.154	0.162	0.019	0.080	0.168	0.202
%CV	0.842	8.811	1.689	0.802	1.878	2.666	1.810	0.985
%Nominal average	103	95	101	101	101	100	103	103

Table 9: Precision and Accuracy batch no-3

Concentration (ng/ml)	Linagliptin				Metformin			
	LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC
Nominal Conc.	1	3	9	20	1	3	9	20
Estimated Conc.	1.032	2.952	9.464	20.305	1.032	3.106	9.279	20.799
	1.021	3.101	9.424	20.422	1.039	3.006	9.395	20.913
	1.038	3.074	9.061	20.515	1.035	2.901	8.972	20.819
	1.085	2.487	9.247	20.930	0.967	2.896	9.247	20.873
Average	1.044	2.904	9.299	20.543	1.018	2.977	9.223	20.851
SD	0.028	0.285	0.184	0.272	0.034	0.099	0.179	0.052
%CV	2.715	9.823	1.983	1.325	3.382	3.341	1.944	0.248
%Nominal	104	97	103	103	102	99	102	104

Table 10: Recovery of LNG and MET

	Linagliptin			Metformin		
	Peak area		(%) Recovery	Peak area		(%) Recovery
	Unex.	Extracted		Unex.	Extracted	
LQC	3608	3180	88.13	7598	6604	86.91
	3913	3249	83.03	6819	6210	91.06
	3983	3229	81.06	7759	6419	82.72
	4251	3290	77.39	7352	6596	89.71
	4594	3835	83.47	7835	6897	88.02
	3783	3275	86.57	7428	6291	84.69
Mean		83.27			87.18	
%CV		4.62			3.56	
MQC	9427	8211	87.10	22098	19805	89.62
	9815	8101	82.53	23240	19812	85.24
	9517	8149	85.62	22562	19719	87.39
	9972	8452	84.75	21492	19218	89.41
	9882	8356	84.55	22437	19201	85.57
	9729	8709	89.51	22562	19652	87.10
Mean		85.67			87.38	
%CV		2.79			2.11	
HQC	25317	21356	84.35	43397	39985	92.13
	26823	21709	80.93	44153	39129	88.62
	26573	21428	80.63	44259	39098	88.33
	26981	20784	77.03	44829	39235	87.52
	26859	21959	81.75	44185	39392	89.15
	26197	20921	79.86	44927	39214	87.28
Mean		80.75			88.83	
%CV		2.96			1.97	
Mean		83.23			87.79	
%CV		2.95			1.02	

Table 11: Recovery of Internal Standard (Phenformin)

IS (Phenformin)			
Sample ID	Peak Area Extracted	Peak Area Unextracted	Recovery (%)
1	30998	30129	97.19
2	31489	30236	96.02
3	31998	29761	93.00
4	31211	25026	80.18
5	31771	28991	91.24
6	31039	29328	94.48
7	32356	29764	91.98
8	31817	29808	93.68
9	31402	29031	92.44
10	31596	29924	94.70
11	31719	29197	92.04
12	31985	29768	93.06
13	31492	28489	90.46
14	31190	28761	92.21
15	31411	28398	90.40
16	31175	28259	90.64
17	31271	28565	91.34
18	31306	28481	90.97
Mean Recovery		92.00	
%CV		3.82	

Table 12: Stock solution stability

Drug	Mean fresh	Mean old stock	Mean % stability
	Stock area	Area	
Short term stock solution stability (after 6 h) (n=3)			
Linagliptin	1043600	1011064	96.88
Metformin	1976500	1918606	97.07
IS	55714	54537	97.88
Long term stock solution stability (after 20 days) (n=3)			
Linagliptin	1016278	931739	91.68
Metformin	1752129	1579421	90.14
IS	51927	49786	95.87

Table 13: Stability data of Linagliptin in plasma

QC Samples Linagliptin	Mean observed at 0 hr	Mean observed at Last	Mean% Stability
Bench top Stability, (after 6 hr) (n=6)			
HQC (20ng/ml)	15451	14791	95.72
LQC (3ng/ml)	2775	2668	96.14
Auto Sampler Stability 12 hr, (25°C) (n=6)			
HQC (20ng/ml)	15398	14492	94.11
LQC (3ng/ml)	2691	2475	91.97
Freeze-thaw Cycle (3 Cycles) (n=6)			
HQC (20ng/ml)	15521	14208	91.54
LQC (3ng/ml)	2290	2107	92.00
Long term Stability (after 20 days) (n=6)			
HQC (20ng/ml)	15927	14197	89.13
LQC (3ng/ml)	2835	2609	92.02

Table 14: Stability data of Metformin in plasma

QC Samples Metformin	Mean observed at 0 hr	Mean observed at Last	Mean% Stability
Bench top Stability, (after 6 hr) (n=6)			
HQC (20ng/ml)	37215	36358	97.69
LQC (3ng/ml)	5410	5309	98.13
Auto Sampler Stability 12 hr, (25°C) (n=6)			
HQC (20ng/ml)	37519	36913	98.38
LQC (3ng/ml)	5257	5134	97.66
Freeze-thaw Cycle (3 Cycles) (n=6)			
HQC (20ng/ml)	37113	35969	96.91
LQC (3ng/ml)	5721	5661	98.95
Long term Stability (after 20 days) (n=6)			
HQC (20ng/ml)	36492	36298	99.46
LQC (3ng/ml)	5574	5413	97.11

Table 15: Dilution integrity data

Specified conc. (ng/ml)	2 Times	
	Calculated conc.(ng/ml)	% Nominal
	Linagliptin	
	481.63	96.33
	510.75	105.15
	517.38	107.48
	487.50	97.50
	467.30	93.46
	532.67	106.53
Avg. conc.		499.53

%CV		4.94
	Metformin	
500 ng/ml	469.37	93.87
	497.03	99.40
	469.61	93.92
	468.43	93.68
	550.14	110.02
	507.25	101.45
Avg. conc.	493.63	
%CV	6.57	

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