Research article

Novel stability indicating LC-MS/MS method for the simultaneous estimation of Remogliflozin etabonate and Vildagliptin human plasma

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ABSTRACT

The present study intended to develop an easy and novel Liquid Chromatography–Mass Spectrometry/Mass spectrometry (LC–MS/MS) method for simultaneous assay of remogliflozin etabonate and vildagliptin, a combined formulation used in treatment of type II diabetes in human plasma. Alogliptin drug was selected as internal standard and the analytes were isolated from the spiked plasma matrix using liquid-liquid extraction procedure and the extracts were chromatographed on Inertsil ODS (4.6 mm×100 mm, 5 μm) C18 column. The mobile phase comprises of methanol, acetonitrile and 0.1 % formic acid in 40:50:10 (v/v) at 0.5 mL/min flow rate and analysis was completed within 6 min run time. The method produces peaks with acceptable symmetry and resolution with acceptable system suitability at 2.6 min for remogliflozin etabonate, 2.7 min for vildagliptin, 1.2 min for alogliptin (internal standard). Multiple Reaction Monitoring (MRM) mode was used for the mass spectral characterization of column eluents using mass detector. In the mass spectral studies confirms the characteristic fragment ion transitions at m/z of 523 to m/z of 247 as MH^+ ion for remogliflozin, m/z of 304 to m/z of 180 as MH^+ ion for vildagliptin. The method can detect both the analyte up to 1.5 ng/mL and having lower limit of quantification (LLOQ) of 5 ng/mL. The method having broad calibration range of LLOQ to 300 ng/mL and was validated for precision, accuracy, stability studies such as freeze-thaw, short term and long-term stability in LLOQ, MQC (medium quality control concentration) and HQC (high quality control concentration) levels and produce acceptable results. Hence it can be confirmed that the method can be adopted for assay of remogliflozin etabonate and vildagliptin simultaneously in plasma samples.

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INTRODUCTION

Remogliflozin etabonate (Figure: 1a) is a prodrug of remogliflozin belongs to gliflozin drug utilised for the treatment of type II diabetes and non-alcoholic steatohepatitis.[1]. Remogliflozin etabonate is the selective Sodium-glucose Cotransporter-2 (SGLT2) inhibitor having advance selectivity and pharmacokinetic (PK) profile among other SGLT2 inhibitors.[2]. It is in single dose was well tolerated and at 1000 mg dosage it increases the urine glucose excretion in healthy subjects whereas at 500 mg dose subjects with type II diabetes mellitus (T2DM). Remogliflozin etabonate reduced plasma glucose concentrations in subjects with T2DM.[3]. Urinary tract infections, dizziness and genital mycotic infections are the common side effects occurred while using the Remogliflozin etabonate.[4].

Vildagliptin (Figure 1b) is the first gliptin of the cyanopyrrolidine class approved for the treatment of type II diabetes

mellitus.[5]. In type II diabetes mellitus patients Vildagliptin not only improve insulin secretion but also suppress the inappropriate glucagon secretion.[6]. Hypoglycaemia, dizziness, headache, nausea and tremor are the vildagliptin side effects. Hepatoxicity was also overserved in rare cases.[7].


Figure 1: Molecular structure of analytes

a) Remogliflozin etabonate   b) Vildagliptin
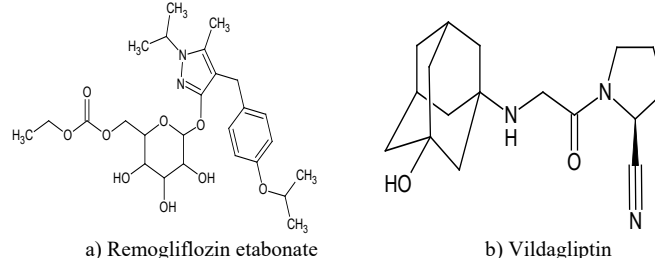
The fixed dose combination of remogliflozin etabonate and vildagliptin improve glycemic control when metformin and one of the

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mono-components of fixed-dose combination do not provide adequate glycemic control, or when already being treated with separate doses of remogliflozin and vildagliptin. The literature survey confirms that few analytical methods available for essay of remogliflozin using HPLC [8,9], UV[10], HPTLC[11] techniques and vildagliptin using HPLC[12-15], UV-visible[16], LCMS[17] and GCMS[18] techniques individually. Few analytical methods reported for assay of remogliflozin in combination with metformin [19-21], vildagliptin in combination with metformin [22-25] and telmisartan [26]. The literature confirms that no method available in open access for assay of remogliflozin etabonate and vildagliptin. Hence, the study was intended to develop LCMS assay method for simultaneous quantification of remogliflozin etabonate and vildagliptin in spiked human plasma. Alogliptin (Figure 2) an anti-diabetic drug was utilised as internal standard.

Figure 2: Molecular structure of alogliptin (Internal Standard)
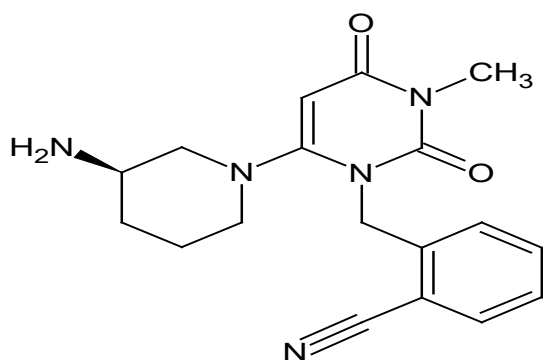
MATERIALS AND METHODS
Equipment and Materials
Waters (Japan) alliance 2695 HPLC having 0. 1 –1500 µL injector capacity auto-injector and is equipped with Waters ZQ (LAA 1369) triple quadrupole mass detector. Masslynx 4.2 (Waters, Japan) software was communicate for spectral intergradation and monitoring the analysis. The pharmaceutically pure remogliflozin etabonate and vildagliptin were obtained from Glenmark Pharmaceuticals Ltd., Secunderabad, Telangana, India. The pharmaceutically pure alogliptin which is selected as internal standard was obtained from Indoco Remedies Ltd, Mumbai, India. The HPLC solvents, formic acid and membrane filters were purchased from Merck chemicals. The plasma of health humans was purchased in a diagnostic laboratory, Guntur, AP.

Standard and sample preparation
The methanol solvent was selected for preparing the standard stock solution at a concentration of 1 mg/mL (1000 µg/mL) for remogliflozin etabonate and vildagliptin separately. The required dilutions of remogliflozin etabonate and vildagliptin were prepared using methanol during the study and internal standard at 100 ng/mL was utilised in the study. Stock solution of remogliflozin etabonate

and vildagliptin was added to plasma to obtain 5, 15, 30, 60, 100, 150 & 300 ng/mL concentrations of both analytes. The internal standard at 100 ng/mL concentration was added all the analyte dilutions.

Sample preparation
The analytes such as remogliflozin etabonate and vildagliptin along with internal standard were extracted from the plasma using liquid-liquid extraction procedure using ethyl acetate solvent. In the liquid-liquid extraction procedure, 100 µL of remogliflozin etabonate and vildagliptin at selected fixed concentration and 100 ng/mL of internal standard were added to the plasma. The precipitation of proteins in the plasma was done by addition 1 mL of ethyl acetate and analytes were extracted using ethyl acetate. The extracted solvent was evaporated and reconstituted with methanol.

Method development
The trails were conducted to separate and analyse the analytes using HPLC. Various method conditions were performed to achieve best results and developed conditions were further validated.

Method validation
The validation of the optimized method was done by following ICH & FDA guidelines [27, 28, 29] and the method parameters like accuracy, reproducibility, selectivity, sensitivity, range/linearity and stability were studied.

Linearity and sensitivity
The linear graph was plotted from LLOQ level and calibration range was assessed after determination of sensitivity of the method. The sensitivity and LLOQ of analytes were determined by analyzing the minimum concentration of analytes that are extracted from spiked plasma containing 100 ng/mL of internal standard. Various dilutions of analytes and 100 ng/mL of internal standard were extracted from spiked plasma and analysed in the developed method. The peak area ratio of analyte to internal standard versus concentration of analyte was used for constructing the calibration curve.

Selectivity and Matrix effect
The chromatographic results achieved for spiked and un-spiked plasma sample were used to evaluate the method selectivity. The chromatographic interference of the blank plasma in the reported method for the separation and detection of remogliflozin etabonate and vildagliptin proves the selectivity and matrix effect of the method.

Precision, accuracy and recovery
The samples at HQC, MQC and LLOQ level in the calibration range was selected for determining the method accuracy and precision. Six independent analyses of the selected concentrations were analysed to evaluate the precision (repeatability) of the method. The % relative standard deviation (RSD) of the analyte to internal standard peak area ratio was calculated and the %

RSD of <2 was considered as precise. The same concentrations were analysed in recovery study and results achieved in this study were compared with aqueous dilutions of the same concentration and the % accuracy of remogliflozin and vildagliptin was calculated. The % accuracy of < 20% in LLOQ range and <15% in other concentrations.

Stability studies

The HQC, MQC and LLOQ levels in the calibration range was selected for stability studies such as freeze thaw, long and short-term stabilities. Short term and long-term stability studies were performed after 24 H incubation at room temperature and – 30 ^{0}C for one month respectively whereas the freeze thaw stability was performed after freeze and thaw cycles. The % stability in all the stability studies was calculated in each studied level.

RESULTS AND DISCUSSION

In the optimization of analytical method for remogliflozin etabonate and vildagliptin various compositions of mobile phase with wide pH range was studied. Based on the chromatographic results observed in each studied trail, the method conditions were confirmed for remogliflozin etabonate and vildagliptin and the conditions were summarized in table 1.

In the finalized method conditions, the chromatogram gives symmetric peaks corresponding to remogliflozin etabonate and vildagliptin as well as alogliptin with undisturbed base line throughout the run time. The retention time was reported at 2.6 min for remogliflozin etabonate, 3.7 min for vildagliptin and 1.2 min for alogliptin. The peak confirmation factors like plate count, tail factor

and the resolution between the analytes were observed to be with in the acceptable limit for all the analytes. The un-spiked sample chromatogram shows no chromatographic detections at the retention time of remogliflozin, vildagliptin and alogliptin confirms that there is no considerable matrix effect identified in the method and the method is specific for the analytes in the study. The chromatogram of un-spiked (3A), analytes and internal standard spiked (3B), internal standard only spiked (3C), remogliflozin spiked (3D) and vildagliptin spiked (3E) chromatograms were given in figure 3.

Table 1: Optimized method development parameters for remogliflozin etabonate and vildagliptin

Condition	Result
Mobile phase	Methano, acetonitrile and 0.1 % formic acid in 40:50:10 (v/v)
Mobile phase pH	5.1
Pump mode	Isocratic
Flow rate	0.5 mL/min
Column	Inertsil ODS (4.6 mm×100 mm, 5 μm) C18 column
Column temperature	Ambient
Detector	PDA and Mass spectrometry (MRM mode)
Injection volume	10 μL
Run time	6 min

The column eluents were identified and characterised using mass spectral analysis using a mass detector and was operated in MRM mode. The accurate and high response of both parent and product ions with high sensitivity was observed in positive ion mode and hence the further analysis was completed by analysing the analytes in positive ion mode.

Figure 3: The chromatogram of blank plasma (3A), standard (3B), individual chromatograms of internal standard (3C), remogliflozin (3D) and vildagliptin (3E)
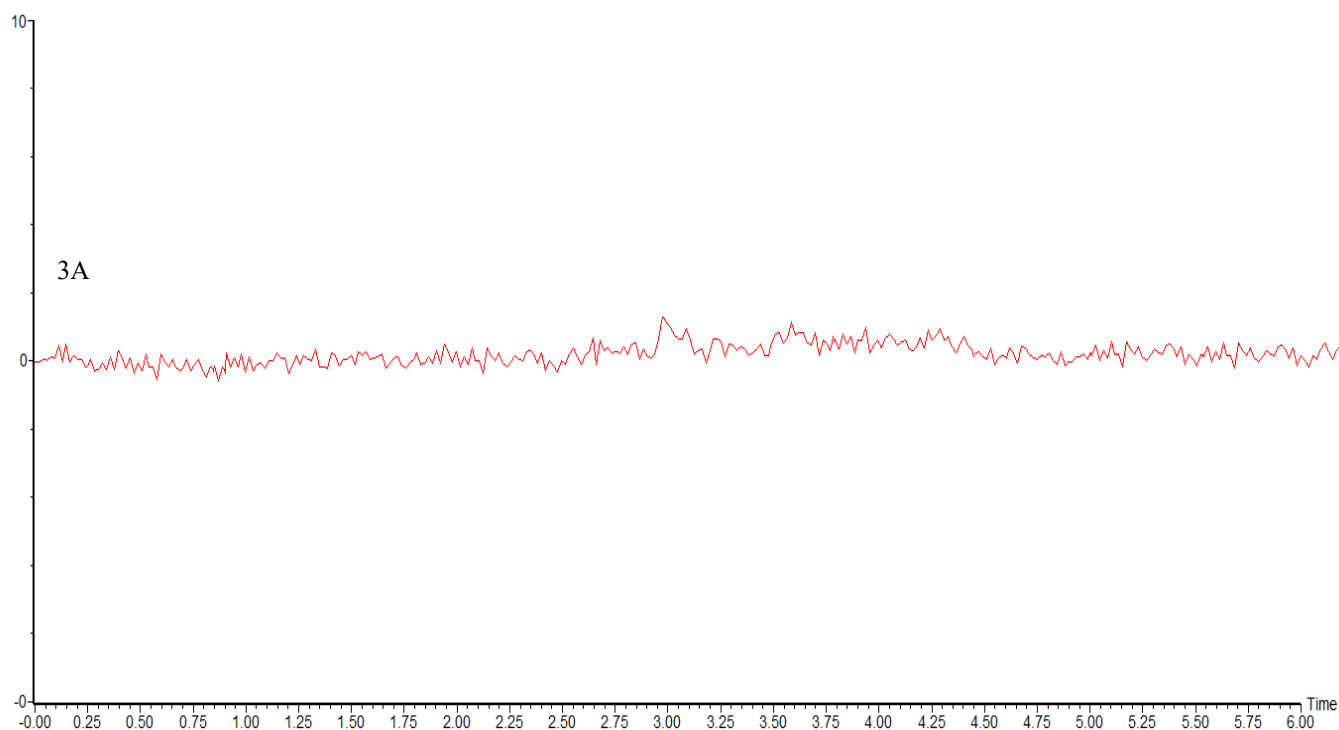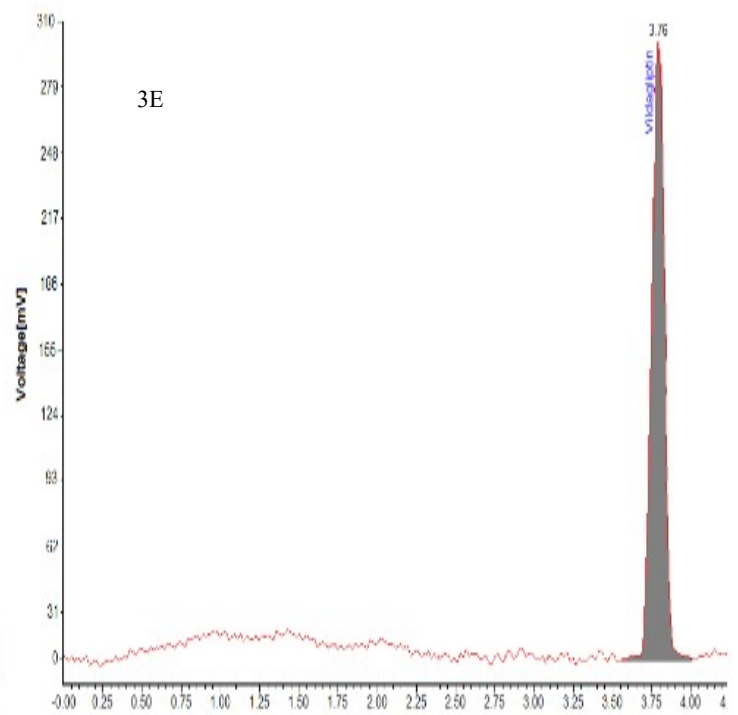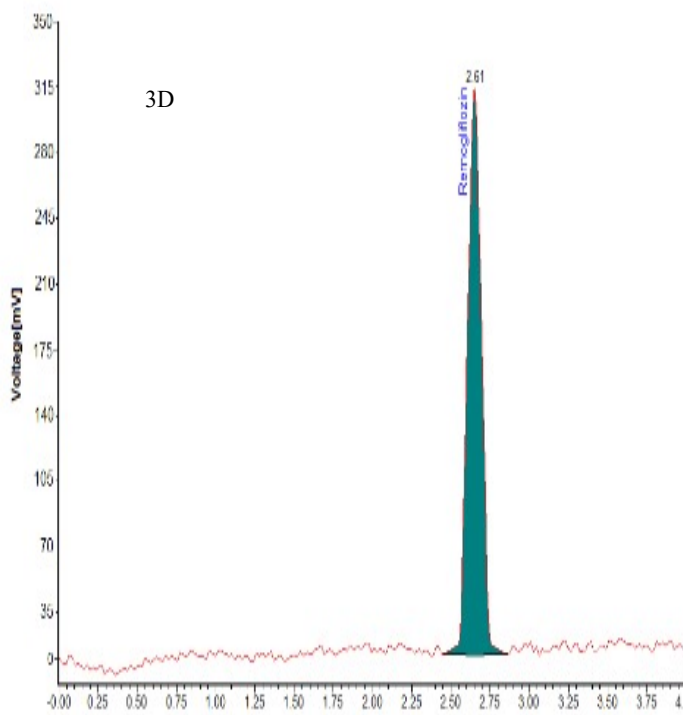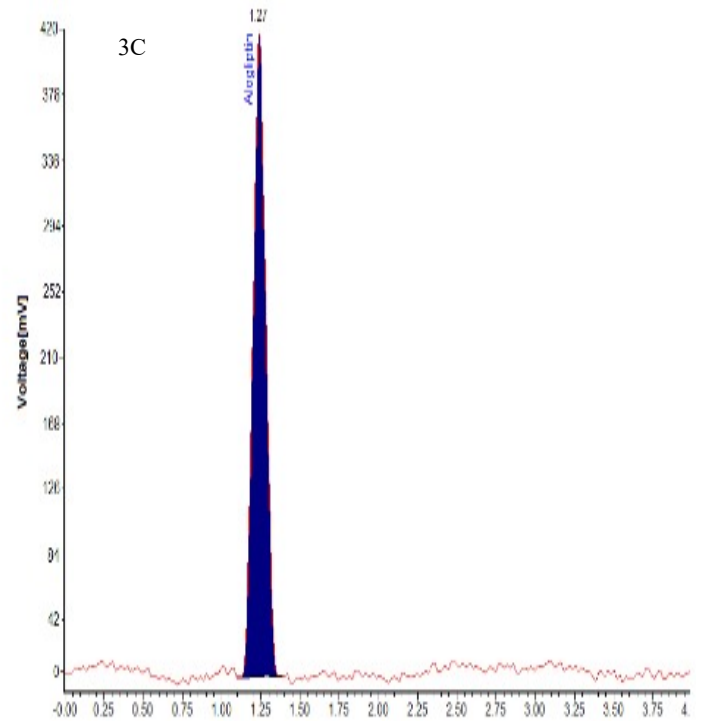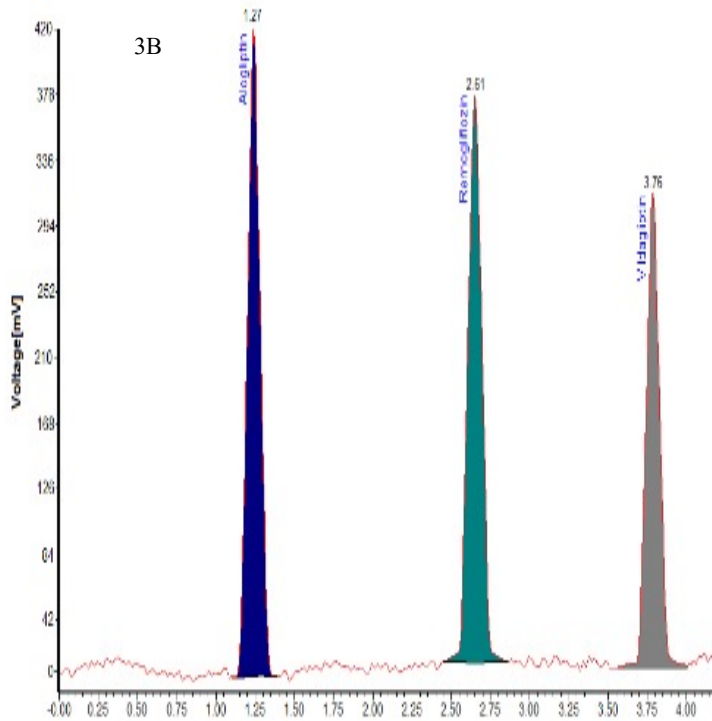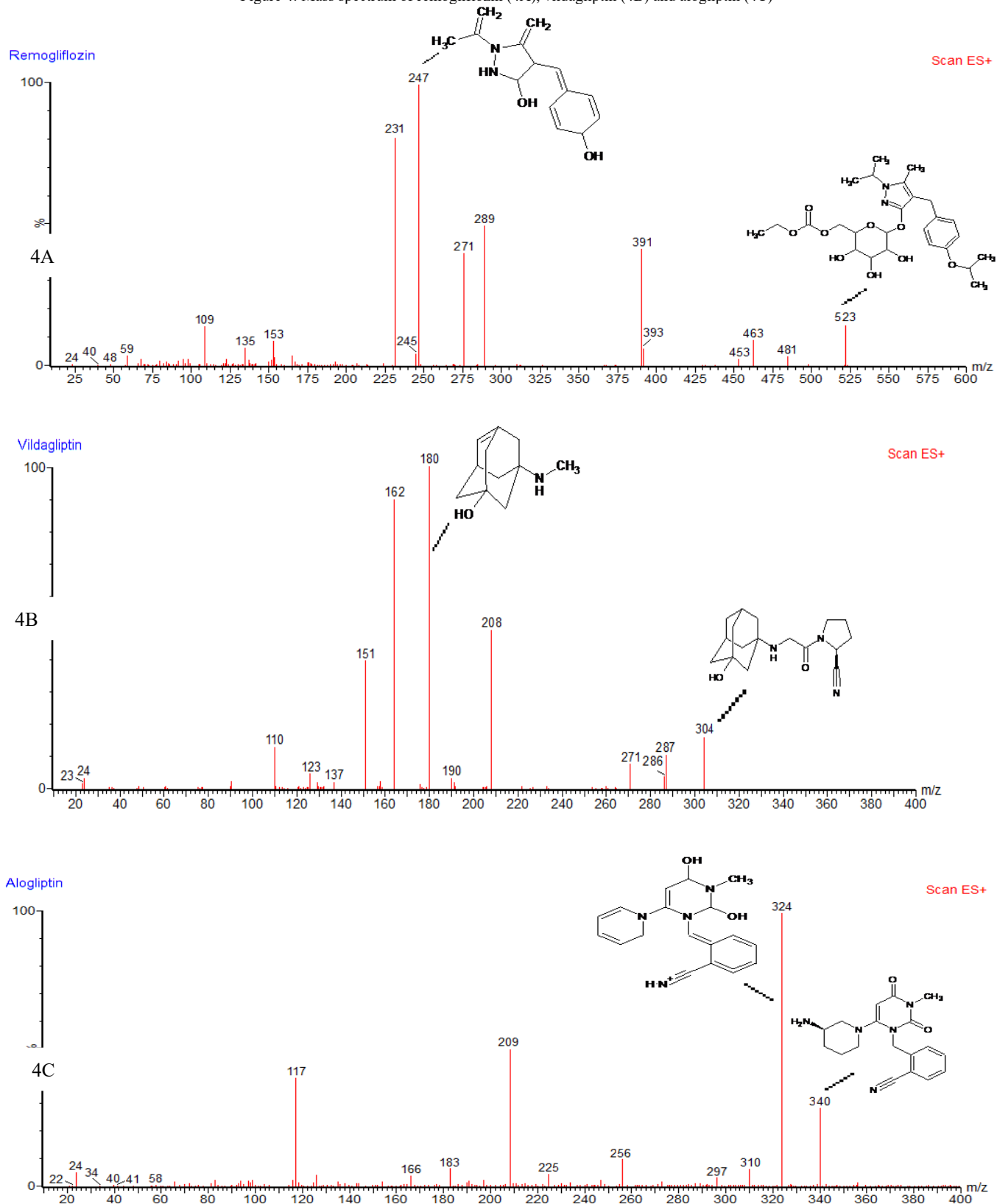
The mass spectra at the retention time of remogliflozin shows parent ion at m/z of 523 which confirms the molecular weight of remogliflozin at positive ion mode (MH⁺). In the mass spectra it is also observed that the characteristic mass fragment at m/z of 247 (MH⁺). The mass spectra of vildagliptin shows parent ion at m/z of 304 which confirms the molecular weight of vildagliptin at positive ion mode (MH⁺). In the mass spectra it is also observed that the characteristic mass fragment at m/z of 180 (MH⁺). Whereas the mass spectra of alogliptin shows parent ion at m/z of 340 which confirms the molecular weight of alogliptin at positive ion mode (MH⁺). In the mass spectra it is also observed that the characteristic mass fragment at m/z of 324 (MH⁺). The mass fragmentation pattern of both the analytes and internal standard was observed to be clear with characteristic fragments corresponds to the parent ion confirms that the method can be applicable for the detection of analytes using mass detector. The mass fragmentation spectra of remogliflozin (4A), vildagliptin (4B) and internal standard (4C) were given in figure 4.

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Figure 4: Mass spectrum of remogliflozin (4A), vildagliptin (4B) and alogliptin (4C)

The LOD was confirmed at 1.5 ng/mL for both remogliflozin etabonate and vildagliptin whereas the LLOQ was calculated at 5 ng/mL in the developed method. In the reported method, the calibration curve was achieved in the concentration limit of 5 – 300 ng/mL for both remogliflozin etabonate and vildagliptin with very high correlation values for both the analytes. The regression equation obtained as $y = 0.0144x + 0.0307$ ($R^2 = 0.9995$) for remogliflozin etabonate and $y = 0.0118x - 0.0014$ ($R^2 = 0.9991$) for vildagliptin (table 2).

Table 2: Linearity results

Concentration. in µg/mL	Peak area			Area ratio of	
	Remogliflozin	Vildagliptin	Internal Standard	Remogliflozin / IS	Vildagliptin / IS
5	29695.8	19868.7	198256.3	0.150	0.100
15	52625.7	38674.8	197858.2	0.266	0.195
30	88686.9	73168.9	198659.2	0.446	0.368
60	175847.5	135257.2	198746.5	0.885	0.681
100	286353.7	223135.8	198556.7	1.442	1.124
150	428326.9	345157.9	198462.5	2.158	1.739
300	871362.8	710251.5	198462.7	4.391	3.579

The % RSD of analyte and internal standard peak area ratio in precision study and % recovery concentration when compared with plasma spiked peak area response ratios to the aqueous concentration analysis results were acceptable proves that method was precise and accurate with high recoveries. The % RSD in each study for remogliflozin etabonate and vildagliptin was within the acceptable (table 3) proved that the method was precise and accurate.

The % stability (table 3) was calculated based on the results observed in stability studies. The results proved that the analytes were observed to be stable in all the three stability studies studied. There is considerable change in the retention time of both analytes as well as internal standard studied. There are no additional detections or the disturbances in the base line was observed through the run time confirms that in all the stabilities studies, the analytes were stable and give reproducible results.

LC-MS is the main method for assay of drugs and their major metabolites in vivo and can provide high enough sensitivity for assay of drugs and their metabolites. In view of this, the present study is intended to develop a sensitive LCMS method for the quantification of remogliflozin etabonate and vildagliptin in spiked human plasma. Similar activity drug alogliptin was selected as internal standard.

The analytes remogliflozin etabonate, vildagliptin and alogliptin were extracted from the spiked plasma using simple liquid-liquid extraction process. The suitable solvent for the extraction of

analytes was confirmed by studying various extraction solvents and its extraction efficiency. The volatile organic solvents such as acetone, ethyl acetate, chloroform, ether were studied and results confirms that ethyl acetate was suitable for extracting the analytes from plasma hence was used throughout the study. The column eluents were identified and characterised using mass spectral analysis using a mass detector and was operated in MRM mode.

The accurate and high response of both parent and product ions with high sensitivity was observed in positive ion mode and hence the same mode was carried in further analysis.

The developed method reports the lowest detection limit of 1.5 ng/mL for both analytes and a sensitive calibration concentration starting from 5 ng/mL and ends up to 300 ng/mL for both analytes. This proves that method having wide and sensitive calibration curves

that having wide applicability for assay of remogliflozin etabonate and vildagliptin.

Table 3: results observed in validation study

Parameter	Results observed	
	Vildagliptin	Remogliflozin
Intraday Precision (% RSD) at		
HQC	0.52	0.69
MQC	0.28	0.35
LLOQ	0.54	0.61
Interday Precision (% RSD) at		
HQC	0.42	0.67
MQC	0.51	0.61
LLOQ	0.61	0.73
Amount (ng/mL) estimated in Freeze and thaw stability at		
HQC	294.37	299.28
MQC	59.31	59.25
LLOQ	4.94	4.92
Freeze and thaw stability (% RSD) at		
HQC	0.44	0.58
MQC	0.40	0.41
LLOQ	0.72	0.59
Amount (ng/mL) estimated in Short-term temperature stability at		
HQC	295.02	295.79
MQC	59.49	59.93
LLOQ	4.94	4.89
Short-term temperature stability (% RSD) at		
HQC	0.68	1.17
MQC	0.94	0.35
LLOQ	0.61	0.44
Amount (ng/mL) estimated in Long-term stability at		
HQC	292.54	293.57
MQC	58.25	58.03
LLOQ	4.81	4.75
Long-term stability (% RSD) at		
HQC	0.57	1.05
MQC	0.87	0.77
LLOQ	0.44	0.57

Finally, the study results were compared with literature, and it can be observed that there is no method reported for assay remogliflozin etabonate and vildagliptin in bulk drug, formulations as well as in biological samples. Hence based on the results achieved and available literature, it can be confirmed that this method will be the best choice for assay of remogliflozin etabonate and vildagliptin in formulations and in biological samples.

CONCLUSION

A sensitive and easy LCMS method was formulated for the assay of remogliflozin etabonate and vildagliptin in biological matrixes such as human plasma. The method comprises a simple and convenient method conditions that can complete an analysis within the shortest run time of 6 min. The method having 1.5 ng/mL detection limit confirms the method sensitivity. The method was validated and all the validation paraments were within the acceptable limit and the analytes were stable in freeze thaw, short term and long-term stabilities. Hence the LCMS method was adequate for the

simultaneous quantization of remogliflozin etabonate and vildagliptin in human plasma as well as bioequivalence studies.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

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