ORIGINAL ARTICLE

Estimation of Remogliflozin Etabonate Active Pharmaceutical Ingredient by Reverse Phase High-Performance Liquid Chromatography Method and Characterization of its Degradation Products by Liquid Chromatography-Mass Spectrometry

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Abstract

Introduction: A reverse phase high-performance liquid chromatography (RP-HPLC) approach that is simple, sensitive, exact, and specific has been developed and verified for Remogliflozin Etabonate (REM) estimation. **Materials and Methods:** Using an isocratic mode methodology, the RP-HPLC procedure was run on a reversed-phase kromasil C18 column (250 mm × 4.6 mm i.d., 5 µm i.d). The detection wavelength was kept at 228 nm and mobile phase consisting of Acetonitrile: 0.1% ortho phosphoric acid in water (60:40). The mobile phase flow rate was maintained at 1 mL/min. For REM, the retention period was 5.5, within the concentration range of 80–120 µg/mL. **Results and Discussion:** The technique showed linearity with a correlation coefficient (r2) of 0.997. The determined limit of detection and limit of quantitation was 0.186 and 0.563, respectively. In accordance with international conference on harmonization Q2 (R1) guidelines, the suggested approach was verified for linearity, accuracy, precision, and robustness. Studies on forced deterioration were conducted to identify a potential degradation mechanism. There was a noticeable difference in the retention period value between the pure drug peak and the degraded product peak, which could be clearly distinguished. The drug was found to be highly sensitive to acid, base hydrolysis, and peroxide conditions. Degraded products were subjected to mass spectral studies and the m/z values were identified for corresponding degradation products. **Conclusion:** The created technique can be applied to regular quality control assessments of REM and stability sample analysis.

Key words: Forced degradation and validation, remogliflozin etabonate, reverse phase high-performance liquid chromatography, SGLT2 inhibitor

INTRODUCTION

Remogliflozin Etabonate (REM) is chemically Ethyl[(2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[5-methyl-1-propan-2-yl-4-[(4-propan-2-yloxyphenyl) methyl]pyrazol-3-yl]oxyoxan-2-yl] methyl carbonate. REM has the molecular weight of 522.6 g/mol and the formula C26H38N2O9.[1] Structure of REM is as shown in Figure 1. Remogliflozin, the active component that inhibits SGLT2, has REM as a prodrug. In diabetic patients, a pathway inhibitor may elevate plasma glucose levels and increase urine glucose excretion. REM is intended to treat type 2 diabetes mellitus (T2DM), both on its alone and in combination with medications that are currently proven to be beneficial.[2]

In literature, Vidhi and Patel showed the ultraviolet (UV) spectrophotometric estimation of REM. This work's objective

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was to create and validate a straightforward, specific, precise, exact, and sensitive UV spectroscopic technique for estimating the dosage of tablets containing REM and its bulk concentration.[3] Sen *et al.* reported UV spectroscopic methods for evaluation of remogliflozin in combination with vildagliptin and metformin in tablet dosage form.[4] Itigimatha *et al.* reported UV spectroscopy and reverse phase high-performance liquid chromatography (RP-HPLC) techniques for measuring REM in pharmaceutical and pure formulation. The results of the proposed techniques showed that the UV spectroscopic and HPLC approaches are straightforward, reliable, and robust for determining REM in dose forms and pure forms. Employing atorvastatin as an internal standard helped develop the procedure.[5] In all the above reports, stability studies were not performed. Shah *et al.* reported study of REM using RP-HPLC method. Studies on forced degradation were conducted to identify a potential degradation mechanism. There was a noticeable variation in the retention period value between the pure drug peak and the deteriorated product peak, which could be clearly distinguished. Here, it was found that the drug was extremely susceptible to basic and acid hydrolysis.[1] Swathi reported remogliflozin sensitivity to acid, base, and peroxide conditions.[6] Several HPLC methods have been developed for the determination of REM with other drugs such as metformin, teneligliptin hydrobromide hydrate, and vildagliptin.[7-10] It has been reported that a stability-indicating high-performance thin-layer chromatography approach for REM estimation in tablet formulation has been developed and validated. Studies on forced degradation conducted here reveal that the drug is extremely susceptible to conditions including acid, base, and oxidative stress.[11] Similarly, survey reveals that few analytical techniques available for the assay of REM using high-performance thin-layer chromatography (HPTLC), a reverse phase ultra-high-performance liquid chromatography/diode array detector (RP-UHPLC/DAD), and ultra-performance liquid chromatography techniques alone or in combination of other drug are also reported.[12-14]

However, there was no research done on the identification and characterization of degradation products. The goal of the current work was to create an analytical RP-HPLC method that is both precise and easy to use by employing forced degradation studies and mass spectrometry to describe the degradation products.

MATERIALS AND METHODS

Instruments and equipment

The Agilent's 1260 Infinity II HPLC system with U.V. detector and PDA detector and software consisting of the OpenLab EZ chrome and the OpenLab, respectively, was used for the study. The Jasco's UV 550 double-beam UV-Visible spectrophotometer with spectra manager software was used as detector. The Aczet CY 224C analytical balance and the bio-technic ultra-sonicator were used for weighing and sonication purpose.

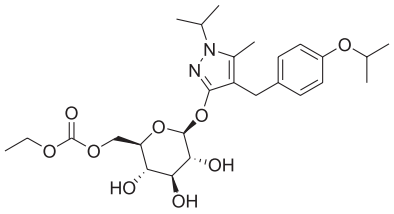

Figure 1: Structure of remogliflozin etabonate

Materials

The complimentary sample of REM was provided by Glenmark Pharmaceuticals Ltd. Chemicals used for analytical study included methanol HPLC grade (Merck), acetonitrile HPLC grade (Merck), water HPLC grade (Siddhi Lab), and ortho-phosphoric acid (OPA) AR grade (Merck).

Methods

Chromatographic condition

The chromatographic analysis used an HPLC system with an Infinity II 1260 Agilent model, a stationary phase with a Kromasil C18 (250 mm × 4.6 mm i.d., 5 µm) column and a mobile phase of acetonitrile: 0.1% OPA in water (60:40% v/v). Before beginning analysis, the LC system was brought to equilibrium with the mobile phase. Eluent was observed at 228 nm using a UV detector, and the flow rate was kept constant at 1 mL/min. Both the column oven temperature and total run duration were maintained at 40°C and 15 min, respectively.

Preparation of stock solution and wavelength selection

Stock solution preparation

After precisely weighing 10 mg of REM, the material was added to a 20 mL volumetric flask. The medication was dissolved by adding 5 mL of methanol and sonicating it. Using methanol, the volume was increased to the required level to provide a standard 500 µg/mL stock solution.

Solution for UV scan

Using methanol, 0.8 mL of the REM stock solution (500 µg/mL) was diluted upto 20 mL (20 µg/mL REM). The REM drug solution and methanol were scanned from 400 nm to 200 nm.

Validation

In compliance with international conference on harmonization guidelines Q2 (R1), the suggested HPLC method was validated for system suitability, solution stability, linearity, specificity, accuracy, precision, intermediate precision, and robustness.[15]

Table 1: Data of linearity

Level (%)	Conc. (µg/mL)	Area	Mean	Standard deviation	% Relative standard deviation
80	80	49499521	49478951	20659.579	0.042
		49458203			
		49479130			
90	90	55563330	55565902	25982.128	0.047
		55593074			
		55541301			
100	100	61594666	61596548	33026.242	0.054
		61630475			
		61564503			
110	110	67586326	67548517	38119.701	0.056
		67549130			
		67510094			
120	120	73617601	73614880	37451.707	0.051
		73650897			
		73576142			

Table 2: Data of accuracy

Level (50%)	Area	Recovered conc.	Added conc.	% Recovery	Mean recovery	% Relative standard deviation
80	49460281	80.314	81.000	99.15	99.15	0.726
	49510734	80.395	80.500	99.87		
	49405095	80.224	81.500	98.43		
100	61609704	100.042	100.500	99.54	99.49	0.625
	61947130	100.590	100.500	100.09		
	61486034	99.841	101.000	98.85		
120	73593905	119.502	121.000	98.76	98.91	0.295
	73571910	119.466	121.000	98.73		
	73651765	119.596	120.500	99.25		

Table 3: Data of precision

Sample	Area	% Assay
Sample 1	61760794	98.80
Sample 2	61248281	98.96
Sample 3	61970431	100.63
Sample 4	61938704	98.60
Sample 5	61560779	99.46
Sample 6	61583710	99.01
Mean		99.24
Standard deviation		0.736876
% Relative standard deviation		0.743

System suitability

A standard stock solution with a concentration of 100 µg/mL was generated for the system suitability study and five runs were taken of the same solution for determination of % relative standard deviation (RSD), theoretical plates, and asymmetry.

Solution stability

It was done at initial (0 h) time point, 12 h and 24 h time point and % absolute difference was calculated.

Specificity

For specificity, the blank and the standard solution were injected to check whether is there any interference in the blank at the retention time of the REM and the standard solution was also injected to check peak purity of the REM. Five levels of working solution were prepared in the range of 80%–120% for linearity study.

Linearity

Linearity graph was plotted and slope, intercept and correlation coefficient values were determined.

Accuracy

Three levels 80%, 100%, and 120% were used to study accuracy. Three copies of each level were prepared. From

each three levels recovered, concentration verses the added concentration was determined and % recovery was calculated.

Precision

Six test samples (each containing approximately 100 µg/mL of REM) were prepared for the precision study, and the percentage of assay and percentage of RSD were assessed. For intermediate precision study, another six sample (about 100 µg/mL of REM) were prepared and % assay, %RSD for 6 reading and % assay, and %RSD for overall reading that is six readings of precision plus six readings that intermediate precision was calculated.

Robustness

Robustness is the ability of the outcome to be maintained even when minor chromatographic circumstances are changed. For this reason, low and high level adjustments were made to the wavelength, mobile phase flow rate, and column oven temperature, and each level's system compatibility was verified.

Limit of detection (LOD) and limit of quantitation (LOQ)

The method's sensitivity was assessed in relation to LOD and LOQ. Formulas 3.3*σ/S and 10*σ/S were used to calculate LOD and LOQ, respectively.

Forced degradation study

Table 4: Data of intermediate precision

Sample	Area	% Assay
Sample 1	61681761	99.17
Sample 2	61904728	100.52
Sample 3	61604785	98.56
Sample 4	61527907	98.92
Sample 5	61547813	99.44
Sample 6	61990378	100.66
Mean		99.55
Standard Deviation		0.8610
% RSD		0.865
Precision plus intermediate precision	Mean	99.394
	Standard Deviation	0.7801
	% RSD	0.785

RSD: Relative standard deviation

Degradation pathways were established and the intrinsic stability of the drug sample is identified with the aid of forced degradation studies. REM was exposed to a variety of stress conditions, including thermal degradation, oxidative hydrolysis, acid-base hydrolysis, and UV light exposure.

Acid hydrolysis

20.1 mg of REM active pharmaceutical ingredient (API) were weighed and then added to a 20 mL volumetric flask. 15 mL of methanol was added, and the API was thoroughly dissolved using a sonicator. Placed the sample on a bench for 24 h after adding 2 mL of 5 N hydrochloric acid (HCl). 2 mL of 5 N sodium hydroxide (NaOH) solution was added to the process to neutralize it after 24 h. Methanol was used to bring volume up (1000 µg/mL). 1 mL of the stock solution was further diluted to 10 mL using mobile phase (about 100 µg/mL of REM) and examined.

Alkali hydrolysis

20.3 mg of REM API were weighed and then added to a 20 mL volumetric flask. 15 mL of methanol was added, and the API was thoroughly dissolved using a sonicator. 0.4 mL of 0.1 N NaOH was added. Kept the sample for 5 min on the bench. The reaction was neutralized after 5 min by adding 0.4 mL of 0.1 N HCl solution. Accomplished volume using methanol. (About 1000 µg/mL of stock). 1 mL of the stock solution was further diluted to 10 mL using mobile phase (about 100 µg/mL of REM) and examined.

Oxidative stress degradation

20.4 mg of REM API were weighed and then added to a 20 mL volumetric flask. 15 mL of methanol was added, and the API was thoroughly dissolved using a sonicator. 2 mL of 30% hydrogen peroxide (H_2O_2) was added. For a 24 h, the sample was kept on the bench. Volume was made up with methanol after a day (About 1000 µg/mL of stock). After diluting 1 mL of the stock solution to 10 mL using the mobile phase (about 100 µg/mL of REM), the results were examined.

Thermal degradation

A sufficient amount of API was placed in a Petri dish, covered with aluminum foil, and punctured with a pointed item. Placed it in a hot air oven set at 60°C for a full 72 h. The sample was removed after 72 h and allowed to come to room

Table 5: Data of robustness

Component	Parameter	Tailing factor (not more than 2)	Theoretical plates (not <2000)
Remogliflozin etabonate	Wavelength: +2 nm	1.08	15183
	Wavelength: −2 nm	1.08	15207
	Flow rate: +10%	1.07	14360
	Flow rate: −10%	1.07	16331
	Column oven temperature: +2°C	1.06	15124
	Column oven temperature: −2°C	1.09	15012

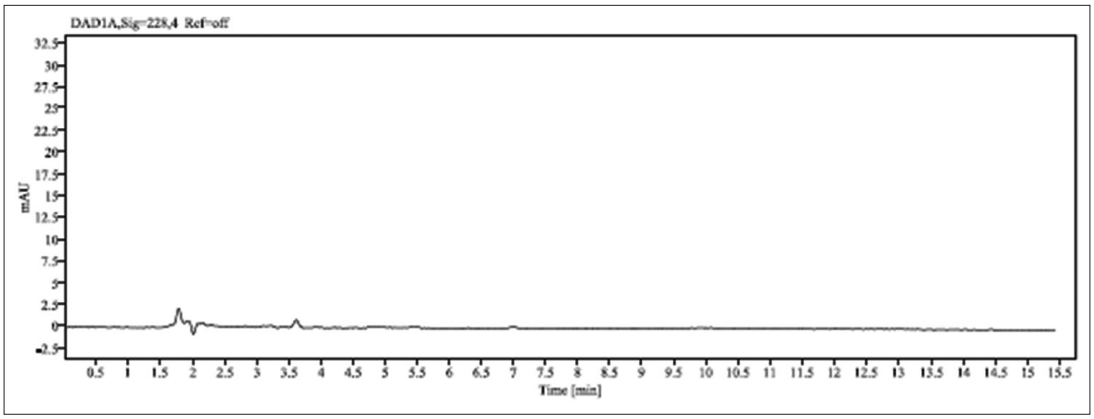

Figure 2: The chromatogram of blank

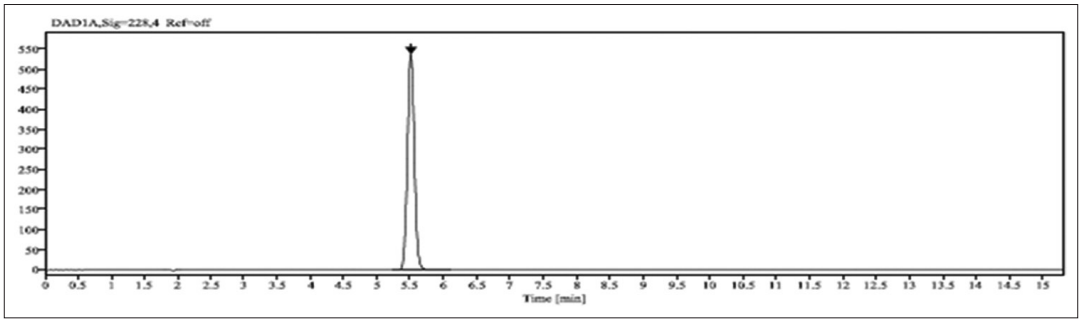

Figure 3: The chromatogram of remogliflozin etabonate with optimized chromatographic conditions.

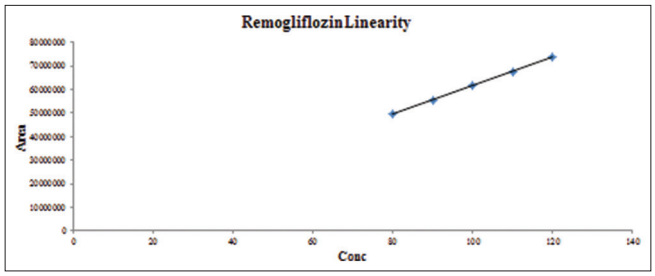

Figure 4: Linearity graph of remogliflozin etabonate

temperature in a desiccator. Subject API prepared using the methodology for preparing samples.

20.2 mg of REM heat-treated API were weighed and then added to a 20 mL volumetric flask. 15 mL of methanol was added, and the API was thoroughly dissolved using a sonicator. Methanol was used to the make up the volume (About 1000 µg/mL of stock). 1 mL of the stock solution was further diluted to 10 mL using the mobile phase to get about 100 µg/mL concentrations and it was examined.

Photolytic degradation

A appropriate amount of API was put in a petri dish, covered with aluminum foil and punctured with a pointed item. Kept for 7 days in the sunlight. The sample was removed after 7 days and placed on a bench top to reach R.T. Subject API prepared using the methodology for preparing samples. 20.1 mg of REM heat-treated API were weighed and then added to a 20 mL volumetric flask. 15 mL of methanol was added, and the API was thoroughly dissolved using a sonicator. Methanol was used to bring the volume up to the mark (about 1000 µg/mL of stock). 1 mL of the stock solution was further diluted to 10 mL using mobile phase (about 100 µg/mL of REM) and examined.

Degradation product characterization

Liquid chromatography-mass spectrometry (LC-MS) analyses were performed on samples that had been strained under the various conditions listed above. Positive electrospray ionization in an MS spectrometer was used to analyze the samples. The HPLC method's same mobile phase worked well with the MS technique. Structure elucidation was performed using the LC-MS data.

RESULTS AND DISCUSSION

Mobile phase optimization

Producing strong peaks for the API with a resolution of more than two and a less asymmetric factor was the aim of the technique development process. To produce a clear and well-defined drug peak, a variety of mobile phases (methanol, water, acetonitrile, and 0.1% OPA in water) were tried. Thus, the mobile phase of the acetonitrile: 0.1% OPA in water (60:40% v/v) ratio was determined to be the best for the duration of the investigation and demonstrated good chromatography. The chromatogram

of blank and the chromatogram of REM with optimized chromatographic conditions is as shown in Figures 2 and 3 respectively.

Method validation

System suitability

For the system suitability, %RSD for the area of five replicates of standard solution was found to be not more than (NMT) 2.0, theoretical plates were found to be not less than 2000 and asymmetry was found to be NMT 2.0. Hence, the system suitability passes all the criteria.

Solution stability

NMT 2.0 was determined to be the percent absolute difference for the solution stability research conducted at the initial, 12-h, and 24-h time points. As a result, it was discovered that the standard solution remained stable for 24 h.

Specificity

The standard solution passed its peak purity after the blank and standard solutions were injected, and the blank showed no interference over the REM retention period. The created chromatographic technique, therefore, satisfied the specificity requirements.

Linearity

The calibration curves were produced by graphing the peak area against concentration with a correlation coefficient of 0.99999 over the 80–120 µg/mL range. Linearity graph and data of linearity of REM is as shown in Figure 4 and Table 1 respectively.

It was discovered that the linear regression equation was y = 602544.73 x + 1306486.6. For the suggested range, the regression coefficient was found to be well within the acceptable limit. The determined values for LOD and LOQ were 0.186 µg/mL and 0.563 µg/mL, respectively.

Accuracy

The preparation of accuracy at each level – 80%, 100%, and 120% – was done in triplicate. The recovered concentration was compared to the added concentration, and the percentage of recovery fell between 98.0 and 102.0%, which is considered acceptable. Data of accuracy is as shown in Table 2.

Precision

The precision test was conducted on six samples (about 100 µg/mL of REM). The individual and the mean assay values were determined to be within the permissible range of 98.0–102.0%, and the percent RSD for the six samples was NMT 2.0. The outcomes are good and repeatable. Data of Precision and data of Intermediate Precision is as shown in the Tables 3 and 4 respectively.

Intermediate precision

For the study on intermediate precision, an additional six samples were prepared. The percentage of assay, the RSD for six readings, and the overall precision were determined by calculating the percentage of assay, the RSD for 12 readings, which consists of six precision readings plus six intermediate precision readings. The results were found to be 98.0 to 102.0% and NMT 2.0, respectively, within acceptable criteria.

Robustness

Variations in wavelength, variations in mobile phase flow rate, and variations in column oven temperature did not affect chromatography. All levels of system appropriateness were found to be within an acceptable range. Thus, the method's robustness can be concluded. Data of Robustness is as shown in Table 5.

Forced degradation study

Acid hydrolysis using 5 N HCl was carried out for 24 h at room temperature, the results showed 84.2% degradation with two degradation peaks at retention time (R.T.) 3.12 and 4.57, respectively, as shown in Figure 5.

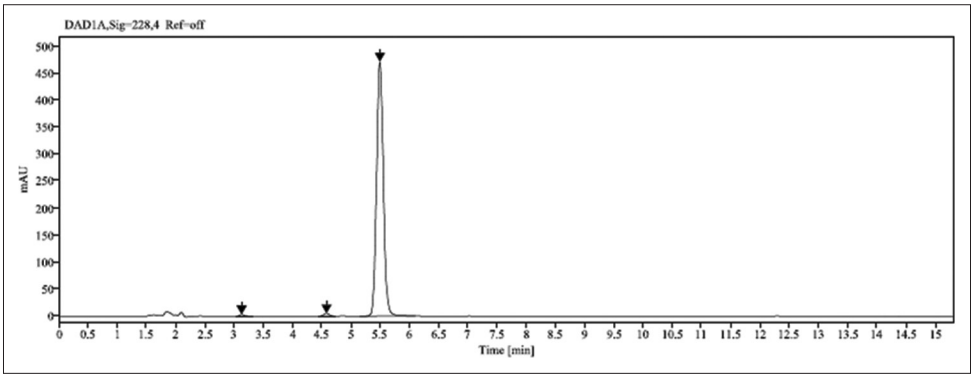

Figure 5: Chromatogram of acid degradation for 2 mL of 5 N hydrochloric acid for 24 h

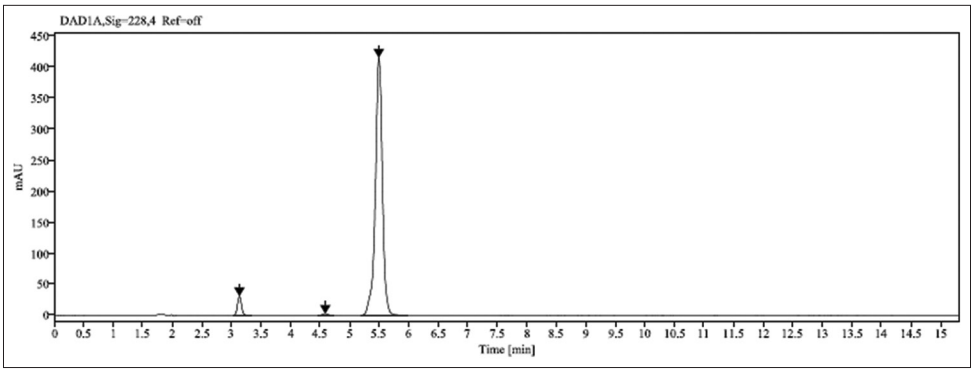

Figure 6: Chromatogram of base degradation for 0.4 mL of 0.1 N NaOH for 5 min

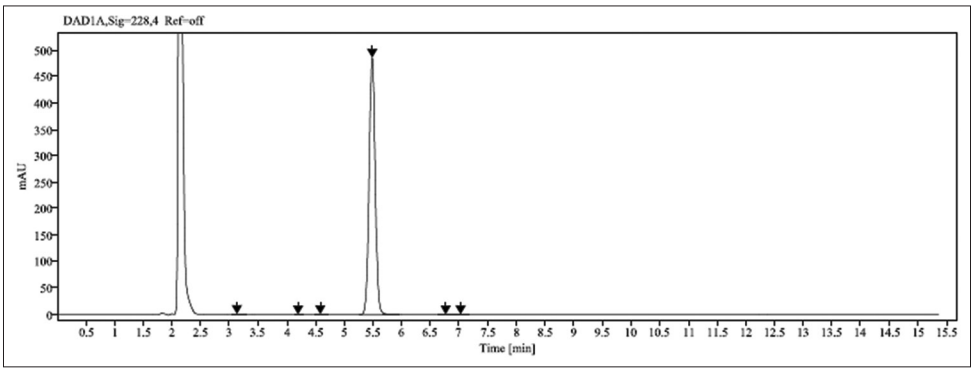

Figure 7: Chromatogram of peroxide degradation for 2 mL of 30% hydrogen peroxide for 24 h

Table 6: Data of forced degradation study

Sample Name	Treatment	Exposure condition	% Assay	% Degradation
active pharmaceutical ingredient	REM	NA	100.00	NA
	Thermal	60°C for 72 h	99.24	0.76
	Photolytic	Sunlight for 7 days	99.37	0.63
	Acid	2 mL of 5 N hydrochloric acid for 24 h at R.T.	91.58	8.42
	Base	2 mL of 5 N NaOH for 24 h at R.T.	0	100
		2 mL of 0.1 N NaOH for 15 min at R.T.	41.99	58.01
		0.4 mL of 0.1 N NaOH for 15 min at R.T.	74.91	25.09
		0.4 mL of 0.1 N NaOH for 5 min at R.T.	87.12	12.88
	Peroxide	2 mL of 30% H_2O_2 for 24 h at R.T.	90.34	9.66

R.T.: Retention time

The results of base hydrolysis using 0.1 N NaOH at room temperature for 5 min revealed 12.88% degradation with two degradation peaks at R.T. 3.13 and 4.58, respectively, as shown in Figure 6.

For the peroxide stress investigation, remogliflozin exhibited 9.66% degradation when 2 mL of 30% H_2O_2 was used for 24 h at room temperature. Five degradation products were found here, and their corresponding R.T. values were 3.12, 4.19, 4.57, 6.76, and 7.02, respectively, as shown in Figure 7.

The drug was found to be stable for thermal and photolytic degradation conditions. The stability data is further summarized in Table 6.

Characterization of degradation product by LC-MS

The column eluates were identified and characterized using mass spectral analysis. Data of forced degradation study by LC-MS is as shown in Table 7.

Acid degradation

REM showed two degradation peaks for acid exposure. Using MS technique for the acid-degradant sample, molecular ion peak for REM, acid degradation product (ADP)-1, and ADP-2 was found at m/z 521.63, 410.34, and 423.888, respectively. Mass spectra of the ADP-1 and ADP-2 are shown in Figures 8 and 10, respectively. Fragments of ADP-1 and ADP-2 are shown in Figures 9 and 11.

Table 7: Data of forced degradation study by LC-MS

Condition	Optimized trial no.	Treatment	% assay	% degradation	Analytes	RT	m/z
Acid treated	Trial no. 1	2 mL of 5 N hydrochloric acid (10%) B.T. for 24 h	91.58	8.42	Remogliflozin	5.49	521.63
					ADP-1	3.12	410.34
					ADP-2	4.57	423.88
Base treated	Trial no. 4	0.4 mL of 0.1 N NaOH (2%) B.T. for 5 min	87.12	12.88	Remogliflozin	5.48	521.47
					BDP-1	3.13	409.89
					BDP-2	4.58	431.18
Peroxide treated	Trial no. 1	2 mL of 30% Hydrogen peroxide (10%) B.T. for 24 h	90.34	9.66	Remogliflozin	5.48	521.40
					PDP-1	3.12	393.63
					PDP-2	4.19	377.20
					PDP-3	4.57	381.52
					PDP-4	6.76	424.15
					PDP-5	7.02	451.05

ADP: Acid degradation product, BDP: Base degradation product, PDP: Peroxide degradation product, RT: Retention time

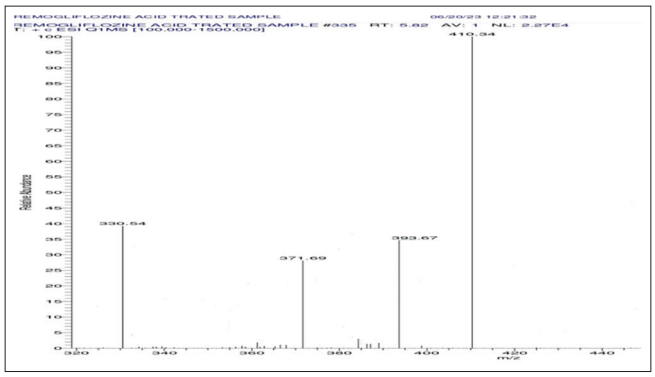

Figure 8: Remogliflozin acid degradation product 1 mass spectra

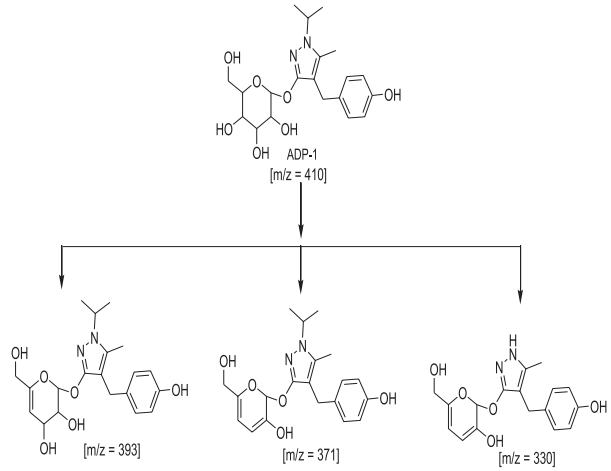

Figure 9: Fragmentation of acid degradation product-1

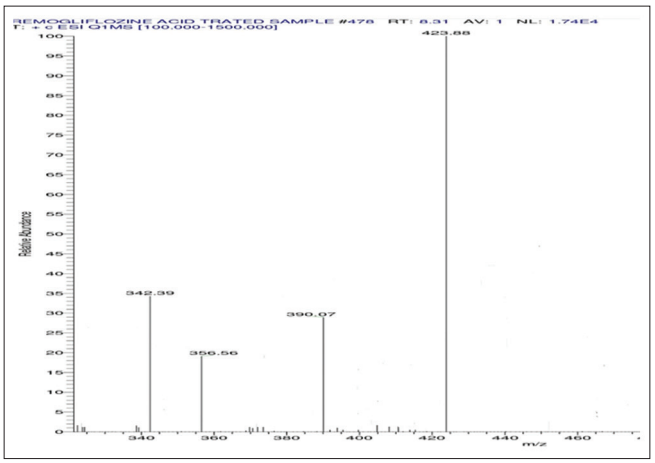

Figure 10: Remogliflozin acid degradation product 2 mass spectra

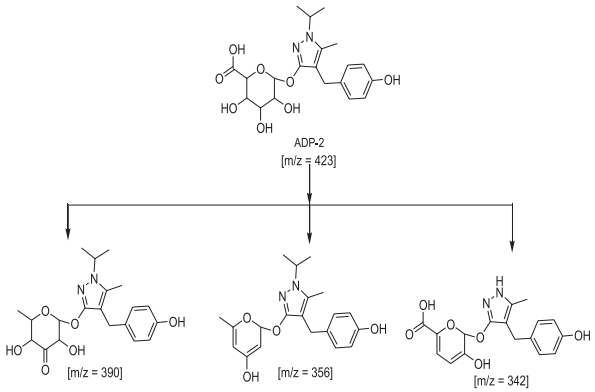

Figure 11: Fragmentation of acid degradation product-2

Alkali degradation

REM showed two degradation peaks for base exposure. Using MS technique for the base-degradant sample, molecular ion peak for REM, base degradation product (BDP)-1, and BDP-2 was found at m/z521.47,409.89 and 431.18, respectively. Mass spectra of the BDP-1 and BDP-2 are shown in Figures 12 and 14, respectively. Fragments of BDP-1 and BDP-2 are shown in Figures 13 and 15.

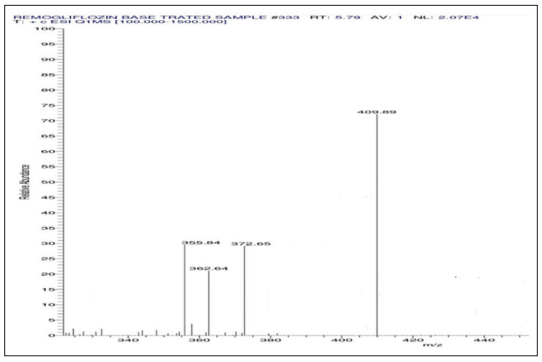

Figure 12: Remogliflozin base degradation product 1 mass spectra

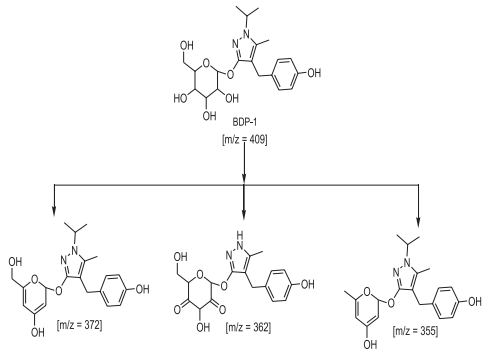

Figure 13: Fragmentation of base degradation product-1

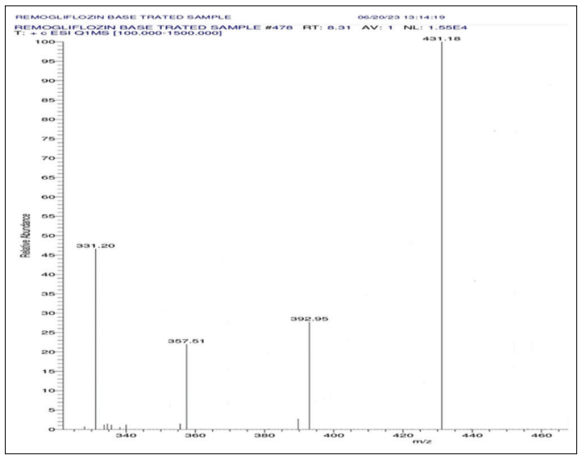

Figure 14: Remogliflozin base degradation product 2 mass spectra

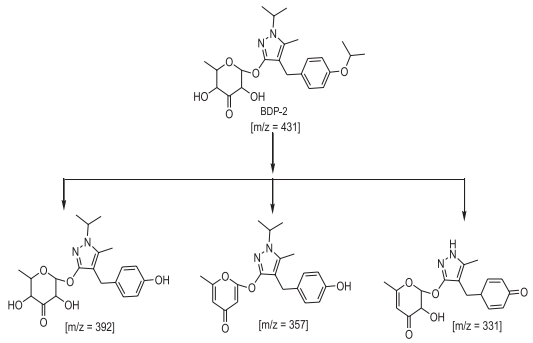

Figure 15: Fragmentation of base degradation product-2

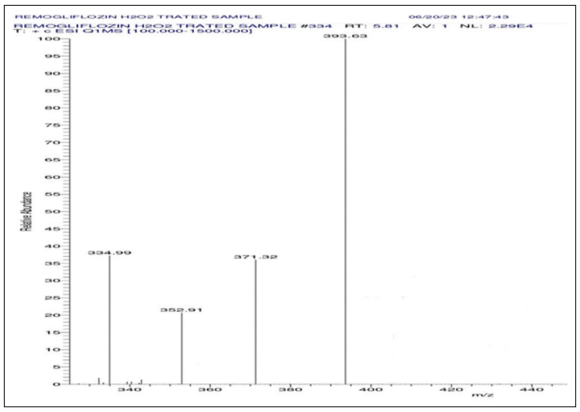

Figure 16: Remogliflozin peroxide degradation product 1 mass spectra

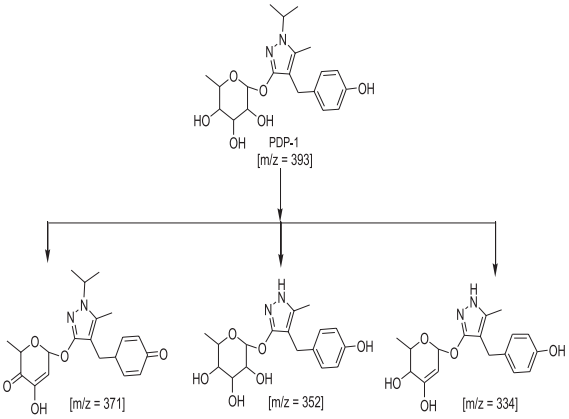

Figure 17: Fragmentation of peroxide degradation product-1

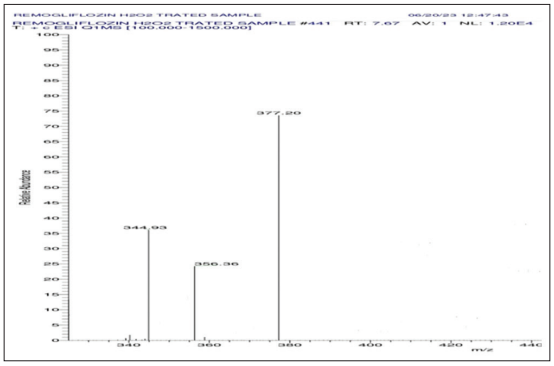

Figure 18: Remogliflozin peroxide degradation product 2 mass spectra

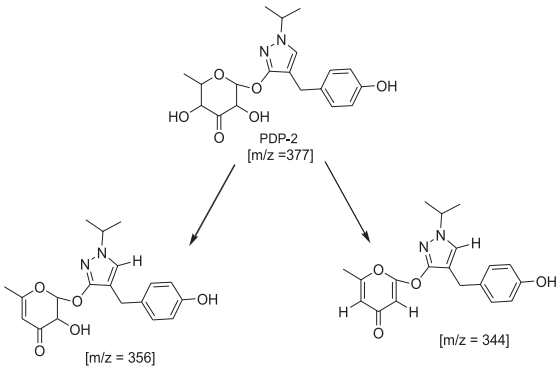

Figure 19: Fragmentation of peroxide degradation product-2

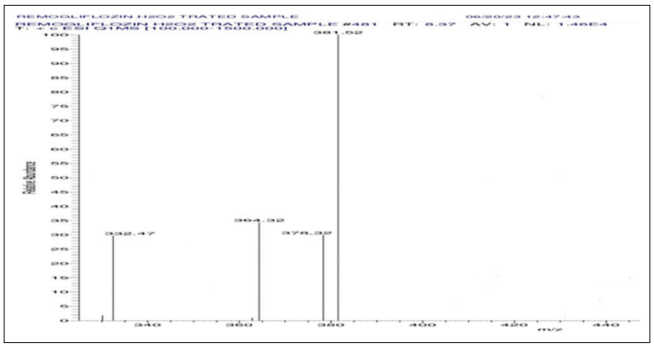

Figure 20: Remogliflozin peroxide degradation product 3 mass spectra

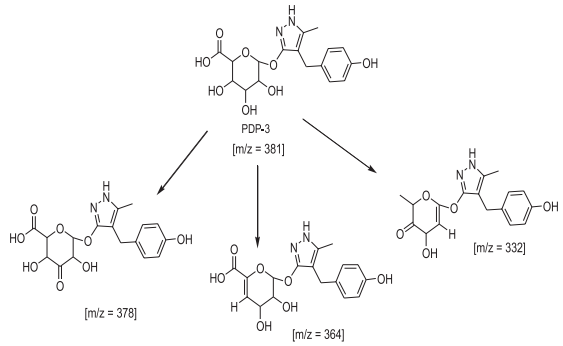

Figure 21: Fragmentation of peroxide degradation product-3

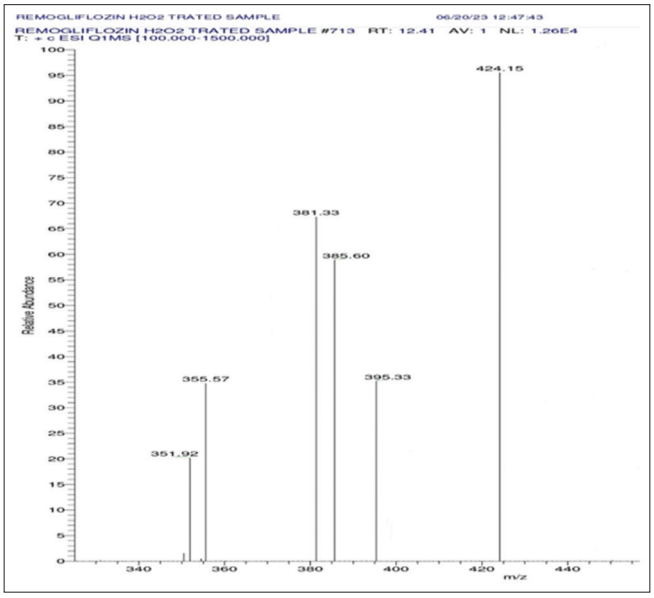

Figure 22: Remogliflozin peroxide degradation product 4 mass spectra

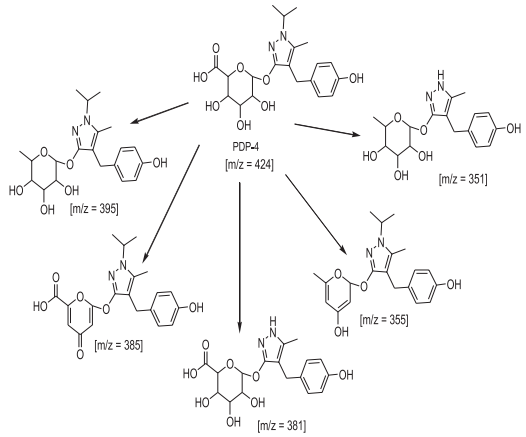

Figure 23: Fragmentation of peroxide degradation product-4

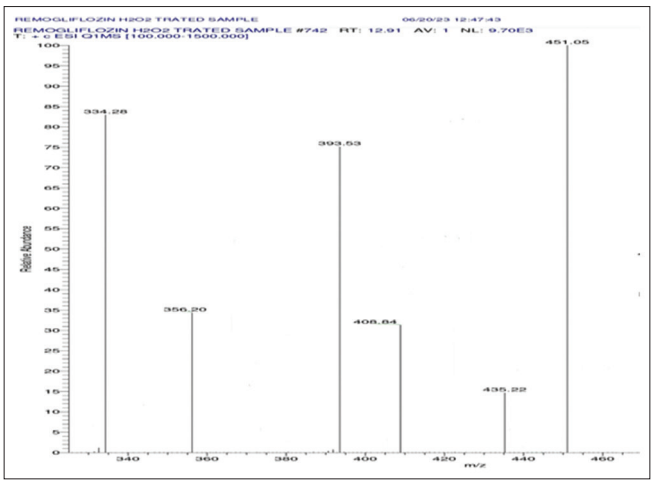

Figure 24: Remogliflozin peroxide degradation product 5 mass spectra

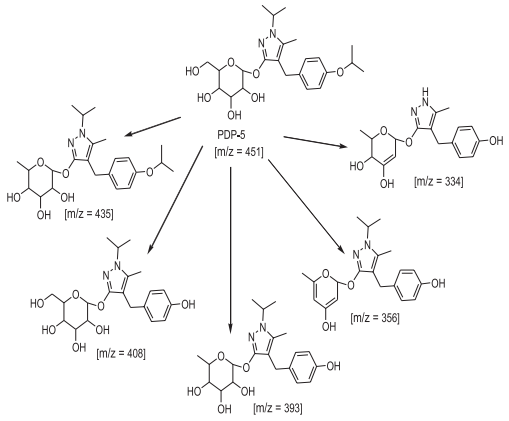

Figure 25: Fragmentation of peroxide degradation product-5

Oxidative degradation

REM showed five degradation peaks for oxidative stress conditions. Using MS technique for the oxidative stressed sample, molecular ion peak for REM, five oxidative degradrant products was found at m/z 521.40, 393.63, 377.20, 381.52, 424.15, and 451.05, respectively. Mass spectra of PDP-1,PDP-2,PDP-3,PDP-4 and PDP-5are as shown in Figures 16,18,20,22 and 24 respectively. Fragments of PDP-1, PDP-2, PDP-3, PDP-4, and PDP-5 are shown in Figures 17, 19, 21, 23, and 25.

CONCLUSION

Using a Kromasil C18 (250 mm × 4.6 mm i.d., 5 µm) column and acetonitrile: 0.1% OPA in water (60:40% v/v) as the mobile phase, the stability-indicating RP-HPLC method was developed for REM API. REM was subjected to a variety of stress conditions in order to conduct forced degradation studies. The REM did, however, display a significant susceptibility to conditions of acid, basic, and peroxide stress, even though it remained stable in the dry heat and photolytic stress conditions. Validation of the procedure revealed that it was sensitive, specific, accurate, and precise. The degradation product formed in forced degradation study was characterized using mass spectrometry.

DATA AVAILABILITY STATEMENT

This article contains all of the data generated or analyzed during this investigation.

ETHICAL APPROVAL

Because in present study, animals were not used as result of this, ethical approval was not required.

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