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LC-MS Based Quantification of Degradents in Pharmaceutical and Bulk Products of Metformin HCl and Remogliflozin Etabonate

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ABSTRACT
The present study focuses on the development and validation of a stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Metformin HCl (MET) and Remogliflozin Etabonate (REM) in pure form and pharmaceutical dosage forms. The analytical method was meticulously designed to effectively separate and quantify the two drugs in the presence of their degradation products under various stress conditions, ensuring its robustness and reliability for stability testing. A C_{18} column was employed for the chromatographic separation, using a mobile phase consisting of a mixture of acetonitrile and phosphate buffer (pH adjusted to 3.0) in a gradient mode. The flow rate was maintained at 1.0 mL/min, and detection was carried out at 235 nm. The method demonstrated excellent linearity over the concentration ranges of 25-150 µg/mL for MET and 2.5-15 µg/mL for REM, with correlation coefficients (r^2) exceeding 0.990 for both drugs. The forced degradation studies, including acidic, alkaline, oxidative, thermal, and photolytic conditions, revealed that the method could effectively separate the degradation products from the intact drugs, confirming its stability-indicating capability. The degradation behavior of both drugs was studied, and the results indicated significant stability under neutral and oxidative conditions, while notable degradation was observed under acidic, alkaline, and photolytic stress. The method was fully validated according to ICH guidelines, demonstrating satisfactory precision, accuracy, specificity, robustness, and system suitability parameters. The method was found to be precise with an RSD of less than 2% and accurate with recovery rates between 98% and 102% for both drugs. In conclusion, the developed RP-HPLC method is highly efficient, specific, and reliable for the simultaneous estimation of MET and REM in pure and pharmaceutical dosage forms, making it suitable for routine quality control and stability testing in industrial and research settings.

Keywords: Metformin, Remogliflozin, RP-HPLC, Simultaneous, Estimation, Forced degradation

INTRODUCTION

Metformin (MET) (**Figure 1A**) is a widely used oral antihyperglycemic agent that is primarily prescribed for the treatment of type 2 diabetes mellitus. It belongs to the biguanide class of drugs and is considered a first-line therapy due to its efficacy, safety profile, and minimal risk of causing hypoglycemia [1]. MET works by reducing hepatic glucose production through the inhibition of gluconeogenesis, increasing insulin sensitivity, and enhancing peripheral glucose uptake, particularly in muscle tissues. This multifaceted mechanism of action helps in lowering blood glucose levels without significantly impacting insulin secretion, thus reducing the risk of hypoglycemia—a common issue with other antidiabetic medications [2]. In addition to its primary role in managing type 2 diabetes, MET has shown potential benefits in other metabolic conditions, such as polycystic ovary syndrome (PCOS), where it improves insulin resistance and helps regulate menstrual cycles. Its use in PCOS is also associated with a reduction in androgen levels, which can alleviate symptoms such as hirsutism and acne [3]. MET is also being explored for its potential in cancer prevention and treatment, particularly in cancers that are associated with metabolic dysregulation, such as breast, prostate, and colorectal cancers. This interest stems from epidemiological studies suggesting that MET users have a lower incidence of certain cancers. The proposed mechanisms include the activation of AMP-activated protein kinase (AMPK), which plays a critical role in cellular energy homeostasis and may inhibit cancer cell proliferation and growth [4]. The drug is generally well-tolerated, with the most common side effects being gastrointestinal in nature, including nausea, vomiting, and diarrhea. These side effects can often be mitigated by initiating treatment at a low dose and gradually increasing it. A rare but serious side effect of MET is lactic acidosis, a condition characterized by the accumulation of lactic acid in the blood, which can be life-threatening if not promptly treated [5]. However, this risk is significantly reduced when MET is prescribed appropriately, particularly by avoiding use in patients with contraindications such as severe renal impairment or conditions predisposing to hypoxia. In clinical practice, MET is available in both immediate-release and extended-release formulations, allowing for flexibility in dosing and enhancing patient adherence. The drug is also

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available in combination with other antidiabetic agents, such as sulfonylureas, DPP-4 inhibitors, and SGLT-2 inhibitors, to achieve better glycemic control when monotherapy is insufficient [6].

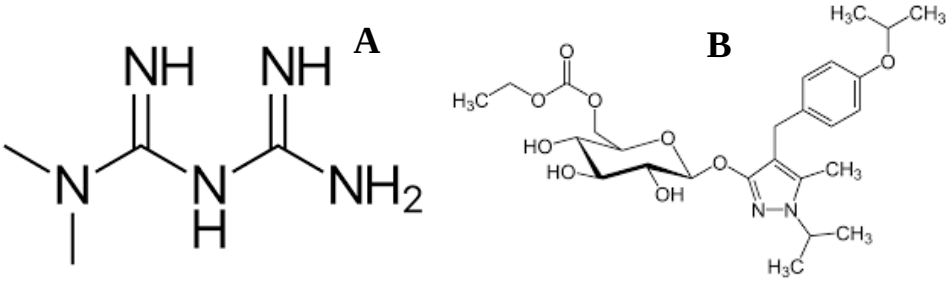

Figure 1. Chemical structure of (A) Metformin (B) Remogliflozin.

Remogliflozin (REM) (**Figure 1B**) is an oral antidiabetic drug belonging to the sodium-glucose cotransporter 2 (SGLT2) inhibitor class. It is primarily used for the treatment of type 2 diabetes mellitus, where it helps to control blood glucose levels [7]. REM works by inhibiting the SGLT2 protein located in the proximal tubules of the kidneys. This inhibition prevents the reabsorption of glucose from the renal filtrate back into the bloodstream, leading to increased urinary glucose excretion and subsequently lowering blood glucose levels. One of the significant advantages of REM, as with other SGLT2 inhibitors, is its insulin-independent mechanism of action [8]. This means that it reduces blood glucose levels without directly affecting insulin secretion or action, making it particularly beneficial for patients with insulin resistance or those at risk of hypoglycemia. Additionally, REM has been associated with weight loss, which is an added benefit for many patients with type 2 diabetes, as obesity is a common comorbidity. Beyond glycemic control, REM and other SGLT2 inhibitors have shown promise in reducing cardiovascular events and protecting kidney function in patients with type 2 diabetes [9]. Clinical studies have suggested that REM can lower the risk of heart failure and slow the progression of diabetic kidney disease, which are common complications of diabetes. This makes REM a valuable addition to the therapeutic arsenal for managing type 2 diabetes, particularly in patients with these comorbid conditions. REM is generally well-tolerated, but as with any medication, it comes with potential side effects. The most common adverse effects are related to its mechanism of action, which includes an increased risk of urinary tract infections (UTIs) and genital mycotic infections due to the presence of excess glucose in the urine [10]. Patients may also experience polyuria (increased urination) and, in rare cases, dehydration. However, the risk of hypoglycemia with REM is relatively low, especially when used as monotherapy. The drug is typically administered once daily and can be used as monotherapy or in combination with other antidiabetic medications, such as MET, to achieve better glycemic control. REM has been studied in various clinical trials, demonstrating its efficacy in lowering HbA1c levels, fasting plasma glucose, and postprandial glucose levels, which are critical markers of long-term glycemic control [11]. REM is also under investigation for its potential benefits beyond diabetes management, such as its effects on non-alcoholic steatohepatitis (NASH) and heart failure. These emerging applications reflect the broader therapeutic potential of SGLT2 inhibitors in treating metabolic and cardiovascular diseases [12].
The literature survey reveals several UV-Vis and RP-HPLC methods for the estimation of MET and REM alone or in combination with other drugs in different dosage forms like tablets, injection, etc preparations. However, no analytical method was reported for the simultaneous estimation of both the MET and REM in bulk and tablet formulation. Therefore, there was a need to develop an HPLC method for the simultaneous estimation of both drugs in both bulk and tablet formulation. The objectives of this research include: (a) Development of RP-HPLC method for the estimation of MET and REM in bulk and tablet formulation; (b) Performing assay of MET and REM; (c) To validate the method developed as per ICH guidelines; and (d) To perform Forced Degradation studies (Acidic, Alkaline, Peroxide, Reduction, Thermal, and Photolytic).

MATERIALS AND METHODS

Materials

HPLC grade solvents such as *ortho* phosphoric acid (OPA), water (Milli Q or equivalent), acetonitrile, ammonium acetate, and hydrogen peroxide were procured from Merck Life Science Pvt. Ltd., Mumbai, Maharashtra, India. Other chemicals like hydrochloric acid, sodium hydroxide, and sodium bisulphate of AR grade were procured from HiMedia Pvt. Ltd., Mumbai, Maharashtra, India. Tablet formulation (Remo-M® 100 mg/1000 mg; labelled claim 1000 mg of MET + 100 mg of REM, manufactured by Glenmark Pharmaceutical Ltd.) were purchased from a local pharmacy shop.

Instrumentation

HPLC (Waters® 2996 with PDA detector) was used to develop and validate the method. The chromatographic separation was carried out by using Hyperclone 5µm BDS C18 130 A°, 150 mm × 4.6 mm, 3.5 µm column, maintained at ambient temperature. Data acquisition was done by using Empower 2 software. The other instruments used were UV-

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Visible Spectrophotometer (Shimadzu® 2203, Kyoto, Japan), and Analytical balance (Shimadzu® AUW220D balance (Kyoto, Japan). Class 'A' Borosil® glassware was employed for volumetric and general purpose in the study.

Mobile Phase

Preparation of Mobile Phase and diluent

A mixture of acetonitrile and buffer in the ratio of 20:80 v/v (filtered through a 0.45 µm membrane filter paper and degassed) and same was used as a diluent throughout the study.

Preparation of Buffer

Weighed accurately about 1.15 g of Ammonium acetate and transferred it into 1000 mL volumetric flask containing 500 mL of HPLC grade water. The content was dissolved by shaking and volume was made upto 1000 mL with water. The pH was adjusted to 3.0 with OPA.

Preparation of Standard Solution

About 100 mg of MET and 10 mg of REM working standard was weighed and transferred into separate 100 mL volumetric flasks. To this, 70 mL of diluent was added and dissolved by sonication for 15 min. The solutions were cooled to room temperature and diluted up to mark with the diluent and used as stock solution (1000 µg/mL of MET and 100 µg/mL of REM). Then, 5 mL of each MET and REM standard stock solutions were transferred into two separate 50 mL volumetric flasks and volume was made up to mark with diluent (100 µg/mL of MET and 10 µg/mL of REM). Similarly the mixed standard stock solution (100 µg/ml of MET and 10 µg/mL of REM) was prepared.

Preparation of Sample Solution

The contents of 20 tablets (Remo-M® 100 mg/ 1000 mg; labelled claim 1000 mg of MET + 100 mg of REM, manufactured by Glenmark Pharmaceutical Ltd.) were weighed, mixed, and average was determined. Weighed accurately about 134 mg of tablet powder (equivalent to 100 mg of MET + 10 mg of REM) and transferred into 100 mL volumetric flask, 50 mL of diluent was added and the content was dissolved by sonication for 30 minutes with intermittent shaking. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. This solution was filtered through 0.45 µm Teflon filter syringe and 5 mL of this solution was further diluted to 50 mL with diluent and mixed.

Assay of formulation

Sample solutions (10 µL) in six replicates were injected and the peak responses were measured and % assay were calculated for MET and REM by using following equation:

$$\%\ \mathbf{Assay} = \frac{\boldsymbol{AT}}{\boldsymbol{AS}} \times \frac{\mathbf{Std.\,wt.\,(mg)}}{\mathbf{10}} \times \frac{\mathbf{1}}{\mathbf{10}} \times \frac{\mathbf{50}}{\mathbf{Wt\ of\ sample\ taken}} \times \frac{\mathbf{50}}{\mathbf{5}} \times \frac{\mathbf{\%\ Potency\ of\ std.\,drug}}{\mathbf{100}} \times \frac{\mathbf{Average\ weight}}{\mathbf{Labeled\ Claim}} \times \mathbf{100}$$

Where,

AT = Average area count of MET & REM peak in the chromatogram of sample solution; **AS=** Average area count of MET & REM peak in the chromatogram of standard solution; **P =** Percent potency of MET & REM working standard on as is basis; and **LC=** Label claim of MET & REM in mg.

Method Validation

Linearity and Range

Linearity for the MET and REM was determined by preparing the standard solutions at six concentrations levels in six replicates levels in the range of 2.5-15 µg/mL for MET and 25-150 µg/mL for REM from the stock solutions. 10 µL of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted. The mean area with its standard deviation and % relative standard deviation of peak areas were calculated. Mean AUC was plotted against concentration to obtain the calibration curve. Regression equations and correlation coefficients were computed from calibration curves [13].

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Blank solution, individual standard solutions and mixed standard solution of MET (100 µg/mL) and REM (10 µg/mL) were injected into the HPLC system. The peak purity data of MET and REM was compared. There should not be any interference at the retention time of the main peaks [14].

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Accuracy

The accuracy study of the method was performed by addition of standard drug to the pre-analyzed sample at three different levels 50%, 100%, and 150% and mean percentage recoveries was determined. Powdered quantity (134 mg) equivalent to 100 mg of the MET and 10 mg of REM was accurately weighed and transferred in three separate 100 ml volumetric flask. To this, 50 mg of MET and 5 mg of REM for 50%, 100 mg of MET and 10 mg of REM for 100% and 150 mg of MET and 15 mg of REM for 150 % of standard MET and REM were added in these three volumetric flask. Each of these was then dissolved in 50 mL of diluent by sonication for 30 min with intermittent vigorous shaking. The solutions were then cooled to room temperature and volume was made up to 100 mL with diluent. The solution was filtered through 0.45 µm Teflon filter syringe and was further diluted with diluent and mixed. The percentage recovery was calculated by using following equation and % RSD should not be more than 2.0 [15].

$$\boldsymbol{Percetage\ Recovery} = \frac{\boldsymbol{A}}{\boldsymbol{B}+\boldsymbol{C}} \times \mathbf{100}$$

Where, **A** = Total drug estimated (mg); **B** = Wt. (mg) of drug contributed by tablet powder; and **C** = Amount of pure drug added (mg)

Precision

System Precision

Six replicates of the mixed standard solution containing the 100 µg/mL of MET and 10 µg/mL of REM were injected into HPLC system. Prepared solutions were analyzed as per the proposed method. The mean, SD, and % RSD were calculated [16].

Method Precision

Six samples containing the known amounts of MET and REM (100 µg/mL & 10 µg/mL, respectively) were analyzed as per test method and the % assay and % RSD for both the drugs was calculated [17].

Intraday and Interday Precision

The intraday precision of the assay method for MET and REM was evaluated at three concentration levels prepared from the sample stock solution (MET 75 µg/mL, 100 µg/mL, and 125 µg/mL & REM 7.5 µg/mL, 10 µg/mL, and 12.5 µg/mL) by performing analysis at an interval of 4 hrs for 12 hrs. The interday precision study was also performed on three different days; *i.e.* day-1, day-2, and day-3 at three different concentration levels as used for intraday study [18].

System Suitability Study

Standard preparations in six replicates (solutions preparation as described in above section) were injected (10 µL). The chromatograms and the peak responses were measured for MET and REM. The system suitability of the method was evaluated in terms of Rt, peak area, tailing factor, resolution, and theoretical plate [19].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ for MET and REM were calculated from slope and standard deviation of the response for MET and REM. The LOD and LOQ were determined using following equations [20]:

$$\boldsymbol{LOD} = \mathbf{3.3} \times \frac{\boldsymbol{\sigma}}{\boldsymbol{S}}$$

$$\boldsymbol{LOQ} = \mathbf{10} \times \frac{\boldsymbol{\sigma}}{\boldsymbol{S}}$$

Where; σ = Standard deviation of response, **S** = Slope of calibration curve

Robustness

Preanalyzed sample solution containing mixture of 100 µg/mL of MET, 10 µg/mL of REM was analyzed as per proposed method by changing the flow rate to 1.2 mL/min and 0.8 mL/min and by changing the mobile phase composition from 20:80 v/v to 18:82 v/v and 20:80 v/v to 22:78 v/v. The system suitability parameters and peak areas (or % assay) was evaluated in each condition and the results were compared with method precision results [21].

Forced Degradation Study

The study was intended to ensure the effective separation of MET and REM from their degradation products in formulation. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method. Forced degradation study was carried out by treating the sample under the following conditions. In order to establish that proposed analytical assay method is stability indicating, sample powder and standard drugs MET and REM individually and their mixture were subjected to the same stress conditions [22,23]. An idea of the origin of the degradation can be obtained by comparing chromatograms obtained for sample, individual drugs and their mixture

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under stress conditions which confirms the stability indicating capacity of the optimized method in both Pharmaceutical substance and Pharmaceutical products. Peak purity study was measured by using purity angle and purity threshold parameters. As the peak purity of MET and REM was well within acceptance criteria for stressed samples. Hence, the method is stability indicating (ICH, 2003 [24]).

Acidic Degradation
Weighed accurately about 134 mg of tablet powder (equivalent to 100 mg of MET and 10 mg of REM) and transferred into 100 mL volumetric flask containing 70 mL of diluent and the content was dissolved by sonication for 20 mins with intermittent vigorous shaking. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. This solution was filtered through 0.45 µm Teflon filter. 5 mL of sample stock solution was transferred in 50 mL volumetric flask, 1 mL of 1 N HCl was added to it and the solution was kept for 1 hr. After 1 hr the solution was neutralized by adding 1 mL of 1 N NaOH. The volume was made upto 50 mL with diluent and mixed. Further, the solution was filtered and injected into HPLC system and the chromatogram was recorded for this solution.

Alkaline Degradation
Weighed accurately about 134 mg of tablet powder (equivalent to 100 mg of MET and 10 mg of REM) and transferred into 100 mL volumetric flask containing 70 mL of diluent and the content was dissolved by sonication for 20 mins with intermittent vigorous shaking. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. This solution was filtered through 0.45 µM Teflon filter. 5 mL of sample stock solution was transferred in 50 mL volumetric flask, 1 mL of 1 N NaOH was added to it and the solution was kept for 1 hr. After 1 hr the solution was neutralized by adding 1 mL of 1 N HCl. The volume was made upto 50 mL with diluent and mixed. Further, the solution was filtered and injected into HPLC system and the chromatogram was recorded for this solution.

Peroxide Degradation
Weighed accurately about 134 mg of tablet powder (equivalent to 100 mg of MET and 10 mg of REM) and transferred into 100 mL volumetric flask containing 70 mL of diluent and the content was dissolved by sonication for 20 mins with intermittent vigorous shaking. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. This solution was filtered through 0.45 µm Teflon filter. 5 mL of sample stock solution was transferred in 50 mL volumetric flask, 1 mL of 3% H_2O_2 was added to it and the solution was kept for 1 hr. The volume was made upto 50 mL with diluent and mixed. Further, the solution was filtered and injected into HPLC system and the chromatogram was recorded for this solution.

Reduction Degradation
Weighed accurately about 134 mg of tablet powder (equivalent to 100 mg of MET and 10 mg of REM) and transferred into 100 mL volumetric flask containing 70 mL of diluent and the content was dissolved by sonication for 20 mins with intermittent vigorous shaking. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. This solution was filtered through 0.45 µm Teflon filter. 5 mL of sample stock solution was transferred in 50 mL volumetric flask, 1 mL of 10% solution of sodium bisulphate was added and sonicated for 1 hr. The volume was made upto 50 mL with diluent and mixed. Further, the solution was filtered and injected into HPLC system and the chromatogram was recorded for this solution.

Thermal Degradation
500 mg of tablet sample was kept at 105°C for 6 hrs in an oven. Weighed accurately about 134 mg this sample powder and was transferred into 100 mL volumetric flask containing 70 mL diluent and the content was dissolved by sonication for 30 minutes with intermittent shaking. Further dilute 5 mL to 50 mL with diluent. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. The solution was filtered through 0.45 µm Teflon filter. Further, 5 mL of sample stock solution was transferred in 50 mL volumetric flask and volume was made up to the mark with diluent. The solution was filtered and injected into HPLC system and the chromatogram was recorded for this solution.

Photolytic Degradation
500 mg of tablet sample was kept at 105°C for 6 hrs in an oven. Weighed accurately about 134 mg this sample powder and was transferred into 100 mL volumetric flask containing 70 mL diluent and the content was dissolved by sonication for 30 mins with intermittent shaking. Further dilute 5 mL to 50 mL with diluent. The solution was cooled to room temperature and the volume was made up to 100 mL with diluent. The solution was filtered through 0.45 µm Teflon filter. Further, 5 mL of sample stock solution was transferred in 50 mL volumetric flask and volume was made up to the mark with diluent. The solution was filtered and injected into HPLC system and the chromatogram was recorded for this solution. The standard solutions of MET (100 µg/mL), REM (10 µg/mL) and mixed standard Solution (100 µg/mL ppm

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and 10 µg/mL) were prepared. These solutions were treated under same stress condition as that of the sample and chromatograms were recorded.

RESULTS AND DISCUSSION

Method Validation

Specificity

There was no interference from the blank at the retention time of analyte peaks. Peak purity data revealed that MET and REM were homogeneous and there were no interference at the retention time of MET and REM peaks. The chromatograms are shown in **Figure 2** and **Figure 3**.

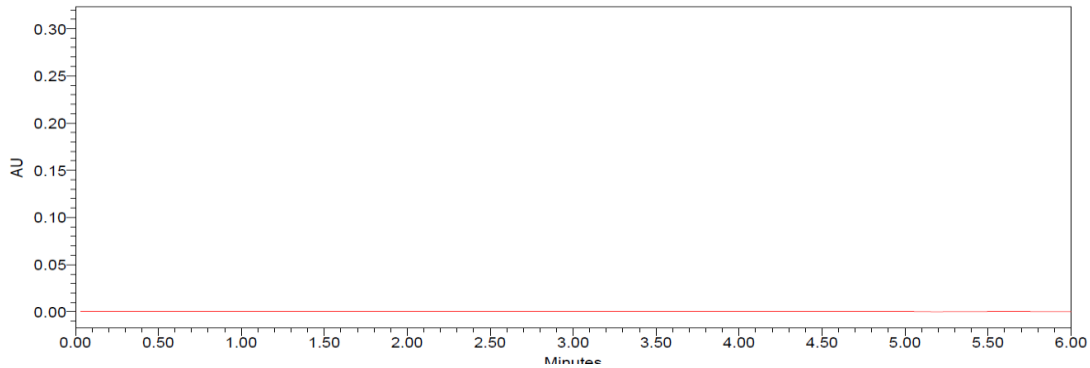

Figure 2. Chromatogram of Blank solution.

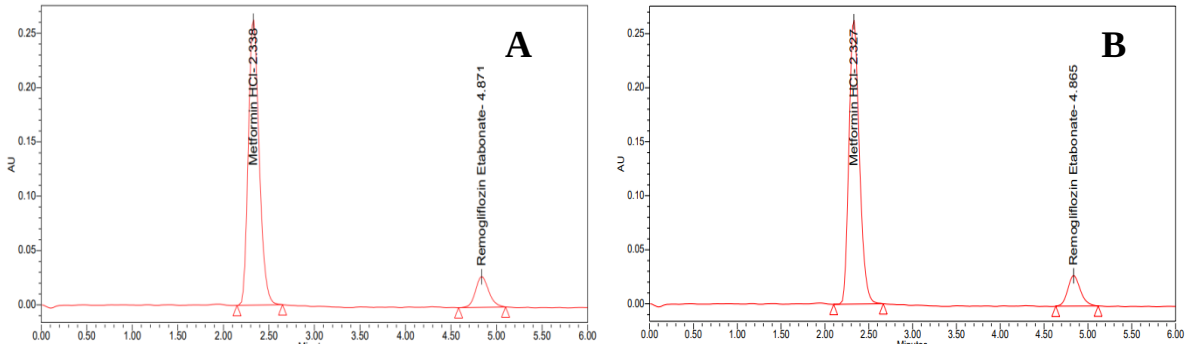

Figure 3. Chromatogram of (A) Standard mixture and (B) Marketed formulation.

Linearity and Range

Linearity of the MET was found in the range of 25-150 µg/mL and REM was found in the range of 2.5-15 µg/mL (**Table 1**). The R^2 value was found to be 0.9997 and 0.9991 for MET and REM (**Figure 4**), respectively. The results showed that an excellent correlation exists between areas and concentration of drugs.

Table 1. Linearity study of MET and REM.

S. No.	Metformin HCl		Remogliflozin etabonate	
	Concentration (µg/mL)	Area	Concentration (µg/mL)	Area
1.	25	607124	2.5	63527
2.	50	1233470	5.0	129458
3.	75	1854721	7.5	197542
4.	100	2462921	10.0	254081
5.	125	3055943	12.5	313240
6.	150	3621295	15.0	375946
Equation of line	**y = 24167x + 24598**		**y = 24800x + 5301**	
R^2	**0.9997**		**0.9991**	

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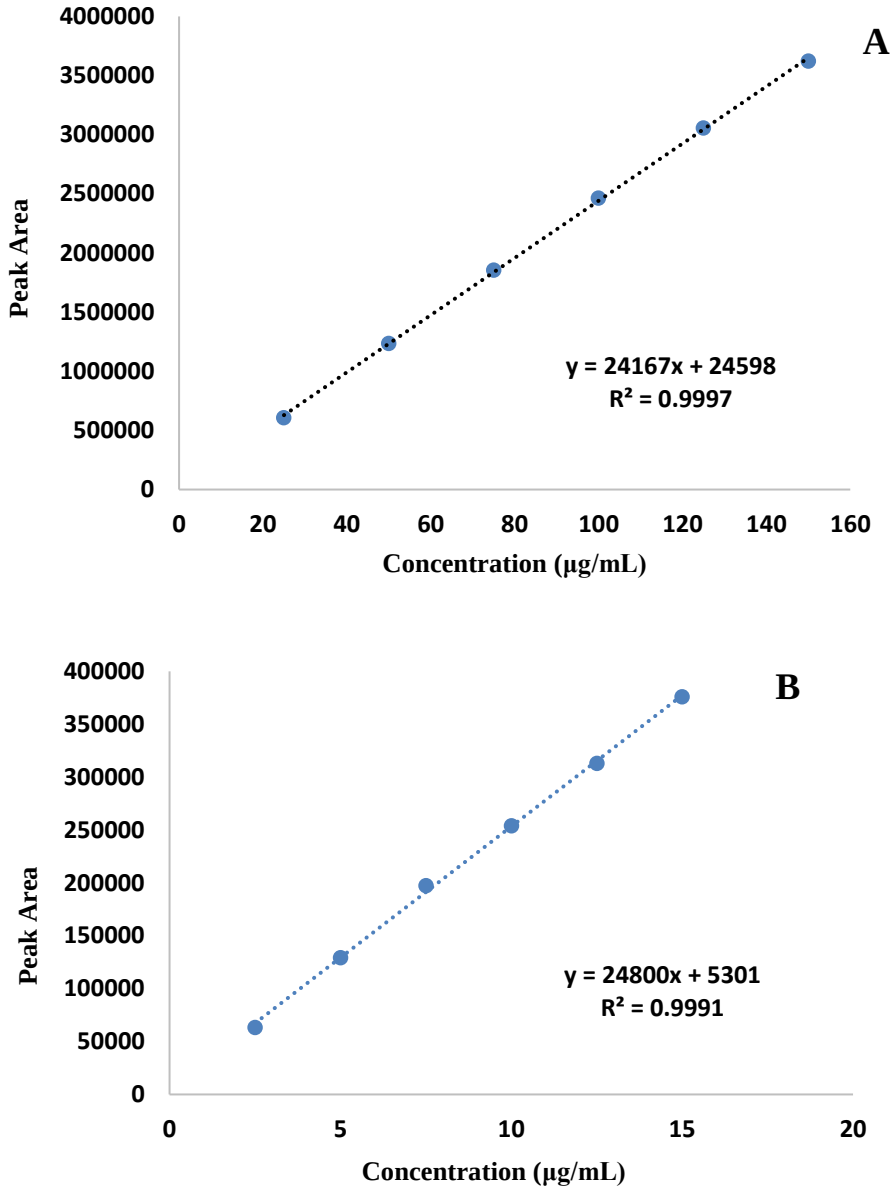

Figure 4. Calibration Curve of (A) MET and (B) REM.

Accuracy

The accuracy of the developed method was determined by performing the recovery of the added standard drug at three different levels 50%, 100%, and 150% and mean percentage recoveries was determined (**Table 2**). The percentage RSD at all the level was found to be in the range of 0.256% w/w - 1.096% w/w which was found well within the acceptable limits (Acceptance limit % RSD should NMT 2).

Table 2. Recovery study for MET and REM.

Statistical Validation of Recovery Studies	**50%**		**100%**		**150%**	
Recovery (%)	**Metformin HCl**	**Remogliflozin etabonate**	**Metformin HCl**	**Remogliflozin etabonate**	**Metformin HCl**	**Remogliflozin etabonate**
Amount Present (mg)	134 mg (100 mg)	134 mg (10 mg)	134 mg (100 mg)	134 mg (10 mg)	134 mg (100 mg)	134 mg (10 mg)
	134 mg (100 mg)	134 mg (10 mg)	134 mg (100 mg)	134 mg (10 mg)	134 mg (100 mg)	134 mg (10 mg)

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	134 mg (100 mg)	134 mg (10 mg)	134 mg (100 mg)	134 mg (10 mg)	134 mg (100 mg)	134 mg (10 mg)
Amount of Std. Added (mg)	50	5	100	10	150	15
	50	5	100	10	150	15
	50	5	100	10	150	15
Amount Recovered (mg)	49.96	5.03	100.89	10.12	149.48	15.16
	49.73	5.08	99.78	10.02	149.95	15.04
	50.36	4.97	99.99	9.96	149.19	15.02
	99.92	100.6	100.89	101.2	99.65	101.07
% Recovery	99.46	101.6	99.78	100.2	99.97	100.27
	100.72	99.4	99.99	99.6	99.46	100.13
Mean Recovery	100.03	100.53	100.22	100.33	99.69	100.49
SD	0.638	1.102	0.590	0.808	0.256	0.505
%RSD	0.637	1.096	0.588	0.806	0.256	0.502

Precision

System precision, method precision, intra precision, and interday precision of the developed method were determined by injecting six replicates of standard solution for system precision and sample solution for method precision into the HPLC system and peak area were recorded for each (**Table 3**). The intraday precision of the developed method was evaluated at 3 concentration levels prepared from the sample stock solution (MET 75 µg/mL, 100 µg/mL, 150 µg/mL & REM 7.5 µg/mL, 10 µg/mL, and 12.5 µg/mL) at an interval of 4 hrs for 12 hrs and on three different days; *i.e.* day-1, day-2, and day-3 for the interday precision (**Table 4**). The % RSD for precision was found in the range of 0.025% - 0.57% which was found well within the acceptable limits (% RSD should not be more than 2).

Table 3. System precision and Method precision.

S. No.	System Precision		Method Precision	
	Metformin HCl	Remogliflozin etabonate	Metformin HCl	Remogliflozin etabonate
1	2468710	252413	99.4	100.3
2	2472441	254589	100.0	99.3
3	2461589	252944	99.9	99.8
4	2465023	253510	99.1	101.5
5	2459785	252735	100.3	101.1
6	2466289	254261	98.8	99.2
Mean	**2465640**	**253409**	**99.6**	**100.2**
SD	**4629.73**	**870.75**	**0.578**	**0.947**
% RSD	**0.188**	**0.344**	**0.58**	**0.95**

Table 4. Intraday Precision and Interday Precision.

Intraday Precision												
				Metformin HCl				Remogliflozin etabonate				
S. No.	Concentration of MET (µg/mL)	Concentration of REM (µg/mL)	Time Interval	% Assay	Mean % Assay	SD (±)	% RSD	% Assay	Mean % Assay	SD (±)	% RSD
1	**75**	**7.5**	After 4 hr	99.978	100.01	0.0251	0.025095	100.03	99.90	0.21	0.208
			After 8 hr	100.028				100.01			
			After 12 hr	100.012				99.66			
2	**100**	**10**	After 4 hr	100.90	100.81	0.0772	0.076561	100.40	100.25	0.53	0.532
			After 8 hr	100.75				99.66			
			After 12hr	100.79				100.70			

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3	**125**	**12.5**	After 4 hr	100.00	99.99	0.01	0.011561	100.01	100.04	0.27	0.272
			After 8 hr	99.97				99.78			
			After 12 hr	99.99				100.32			
		Interday Precision									
1	**75**	**7.5**	Day-1	100.00	100.03	0.02687	0.026879	99.48	99.42	0.5670413	0.57
			Day-2	100.05				99.96			
			Day-3	100.04				98.83			
2	**100**	**10**	Day-1	100.30	100.73	0.40415	0.401203	99.70	99.79	0.1884436	0.189
			Day-2	100.80				99.67			
			Day-3	101.10				100.01			
3	**125**	**12.5**	Day-1	99.98	99.99	0.03273	0.032735	100.41	100.07	0.3087892	0.309
			Day-2	99.97				100.01			
			Day-3	100.03				99.80			

System Suitability Study
Standard solutions in five replicates were injected and system suitability parameters were evaluated in terms of Rt, peak area, tailing factor, resolution, and theoretical plate (**Table 5**). All the system parameters were found well within the acceptable limits.

Table 5. System Suitability Parameters.

	System Suitability Parameters				
	Retention Time (Min)	**Area (mV)**	**Resolution**	**Tailing factor**	**Plate Count**
Metformin HCl	2.335 (±0.036)	2465639.5 (±4629.73)	NA	1.160 (±0.030)	14315.16 (±283.12)
Remogliflozin etabonate	4.875 (±0.003)	253408.667 (±870.749)	10.238 (±0.027)	1.058 (±0.030)	9641.83 (±20.702)
Acceptance Criteria	--	--	**NLT2**	**NMT2**	**NLT 2000**

*Average of six determinations; NA: Not Available

Robustness
Preanalyzed sample solution containing mixture of 100 µg/mL of MET, 10 µg/mL of REM was analyzed as per proposed method by changing the flow rate and by changing the mobile phase composition. The system suitability parameters and peak areas (or % assay) was evaluated in each condition and the results were compared with method precision results. All the system suitability parameters were found well within the acceptance limit indicates that the developed method is robust for the determination of both the drug simultaneously.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)
LOD and LOQ for MET was found to be 0.30 µg/mL and 1 µg/mL and for REM 0.03 µg/mL and 0.1 µg/mL, respectively.

Assay
Six replicates of 10 µL of the sample were injected into the HPLC system and the areas for both MET and REM peak were measured and calculated the % assay. The average % assay should be within range of 98-102% and were found to be 99.1% w/w for MET and 100 % w/w for REM, which are well acceptable in limit (**Table 6**).

Table 6. Assay of the Marketed formulation.

Marketed formulations and Strength	**RT of MET**	**Average Peak Area**	**% Assay of MET**	**RT of REM**	**Average Peak Area**	**% Assay of REM**
Remo-M 100/1000 containing 1000 mg of MET + 100 mg of REM	2.325 (±0.00424)	2443339 (±7673.52)	99.10 (±0. 0.3112)	4.864 (±0.00424)	253232 (±1076.21)	100.00 (±0.4249)

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*Average of Six Determinations

Forced degradation studies

The forced degradation studies at each condition were carried out for the sample, standard solutions of MET, REM and mixed standard Solution.

In acid treated sample the percentage assay and degradation of MET and REM was found to be 86.5% w/w, 13.5% w/w and 89.2% w/w, 10.8% w/w, respectively. In alkali treated sample the percentage assay and degradation of MET and REM was found to be 96.6% w/w, 3.4% w/w and 97.7% w/w, 2.3% w/w, respectively. In peroxide degradation of sample the percentage assay and degradation of MET and REM was found to be 85.9% w/w, 14.1% w/w and 86.6% w/w, 13.4% w/w, respectively. In reduction degradation of sample the percentage assay and degradation of MET and REM was found to be 89.6% w/w, 10.4% w/w and 96.0% w/w, 4.0% w/w, respectively. In thermal degradation of sample the percentage assay and degradation of MET and REM was found to be 97.5% w/w, 2.5% w/w and 89.7% w/w, 10.3% w/w, respectively. In photolytic degradation of sample the percentage assay and degradation of MET and REM was found to be 96.9% w/w, 3.1% w/w and 96.5% w/w, 3.5% w/w, respectively.

Both the drugs showed more degradation in the acidic and peroxide degradation studies. The percentage assay, percentage degradation at each condition along with their purity angle and purity threshold for MET and REM are tabulated in the **Table 7**. Forced degradation studies were performed on standard drug alone, mixture of standard drugs, and their formulation. The degradants products in individual drug, standard mixture and formulation were compared and it was found that degradation product in formulation were same as that in individual drugs. From the same retention time it is clear that same degradation products are formed in the formulation and in pure drugs under identical stress conditions.

Table 7. Forced Degradation Study of MET and REM in the sample.

		Peak Purity for Metformin HCl peak				**Peak Purity for Remogliflozin etabonate Peak**			
S. No	**Condition**	**% Assay of MET**	**% degradation w.r.t. Control**	**Purity Angle**	**Purity Threshold**	**% Assay of REM**	**% degradation w.r.t. Control**	**Purity Angle**	**Purity Threshold**
1	**Control Sample**	100	0	1.242	4.044	100	0	0.784	5.842
2	**Acid degradation**	86.5	13.5	1.22	4.072	89.2	10.8	0.742	5.878
3	**Base degradation**	96.6	3.4	1.269	4.043	97.7	2.3	0.738	5.833
4	**Peroxide degradation**	85.9	14.1	1.215	4.068	86.6	13.4	0.717	5.885
5	**Reduction degradation**	89.6	10.4	1.207	4.059	96	4	0.756	5.801
6	**Thermal degradation**	97.5	2.5	1.243	4.087	89.7	10.3	0.709	5.816
7	**Photolytic degradation**	96.9	3.1	1.232	4.095	96.5	3.5	0.754	5.851
8	**Hydrolytic degradation**	98	2	1.212	4.003	97	3	0.736	5.819

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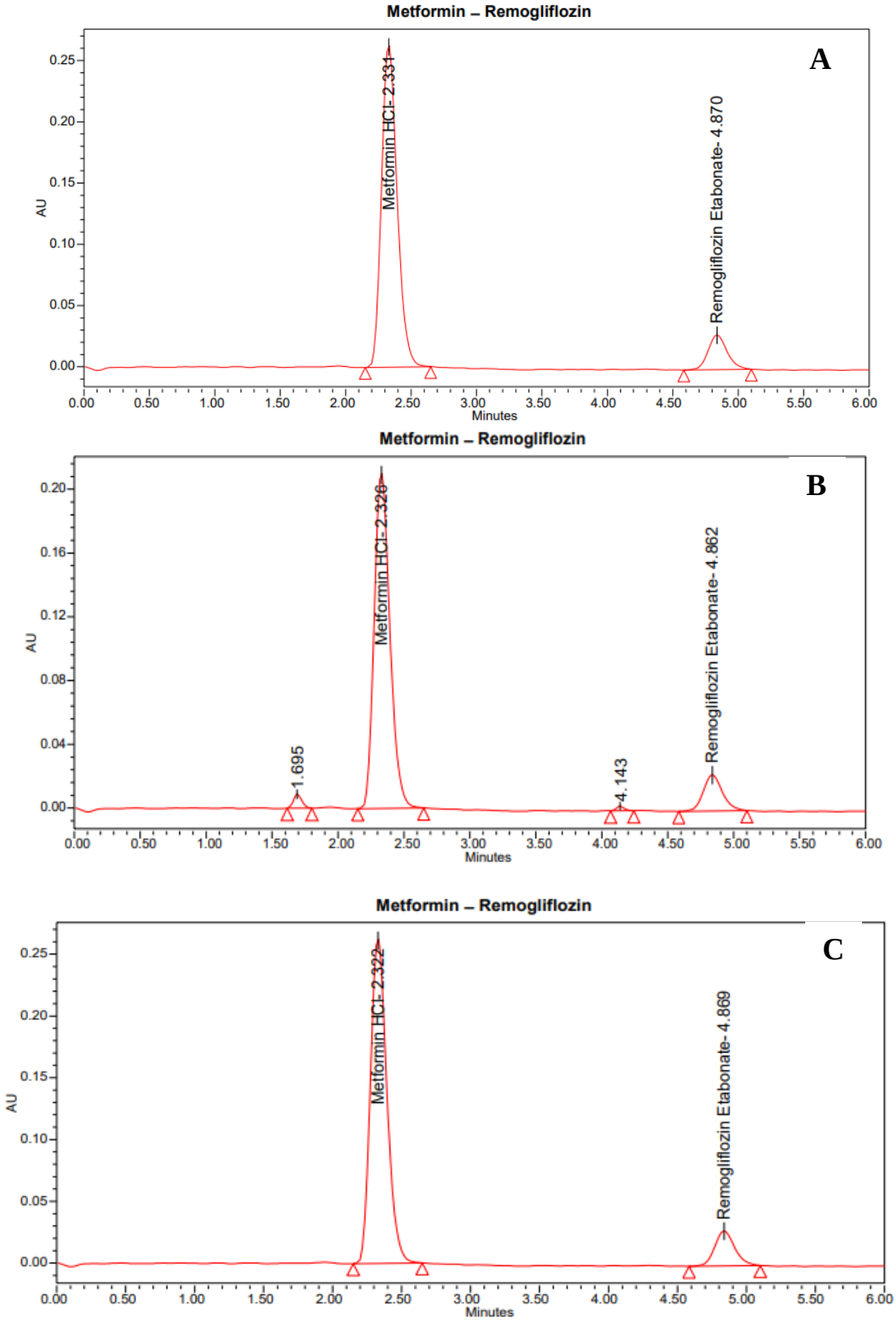

Figure 5. Chromatogram of sample: (A) Control, (B) Acidic Degradation, and (C) Alkaline Degradation.

Peak Purity plot

The Peak Purity plot plays a vital role in determining the integrity and specificity of the peaks during the HPLC analysis. Peak purity indicates whether a chromatographic peak consists of only one component or if it's co-eluting with

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other substances, which is critical when evaluating degradation products under stress conditions like acidic and alkaline degradation.

In the controlled sample (**Figure 6A**), MET and REM should produce well-defined, symmetrical peaks with high peak purity. The peak purity plot will show a straight line close to unity, confirming that the peaks represent a single component with no interference from impurities or degradation products. Both MET and REM is stable under normal conditions, and their retention times are consistent with the method parameters.

Under acidic degradation conditions (**Figure 6B**), MET and REM are subjected to hydrolysis, which may lead to the formation of degradation products. The peak purity plot in this case might show a deviation from unity, indicating co-elution or overlapping peaks, which confirms the presence of degradation by-products. For MET, a slight shift or additional peaks might appear around its retention time due to its susceptibility to acidic hydrolysis. REM, being more sensitive to acidic environments, is expected to show a more significant drop in peak purity, with the emergence of degradation peaks in the chromatogram. The peak purity index will decrease if these degradants co-elute with the main drug peak, signifying a loss in specificity.

Similarly, in alkaline degradation (**Figure 6C**), both MET and REM are exposed to basic conditions, leading to potential breakdown into smaller fragments or degradation products. The peak purity plot under alkaline conditions would again reveal any co-elution or impurity presence. For MET, alkaline hydrolysis is less severe compared to acidic conditions, so the peak purity plot might still remain close to unity, with only minor changes. However, REM, being prone to alkaline degradation, will show more prominent additional peaks, and the peak purity might drop significantly, signaling substantial degradation.

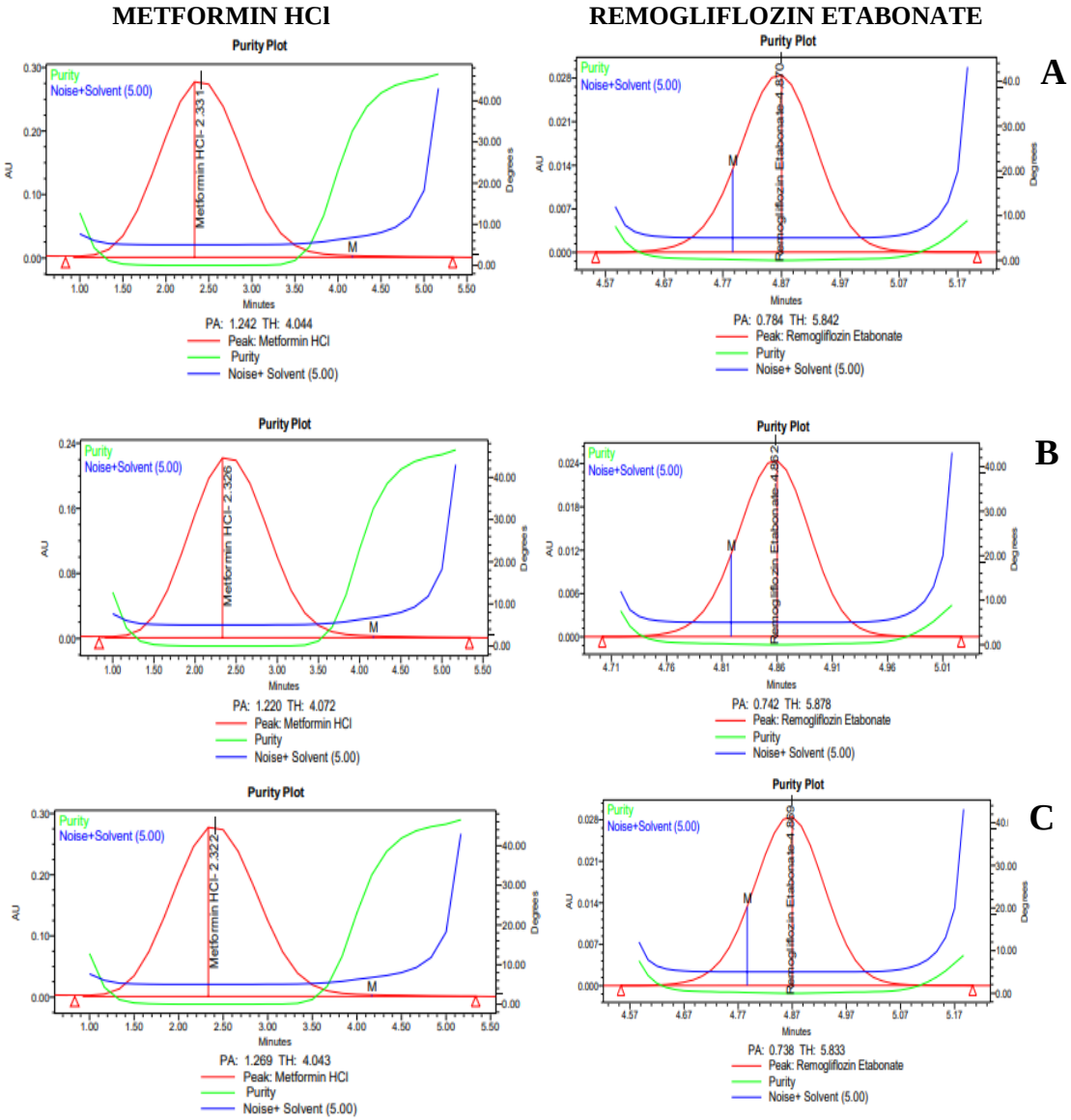

Figure 6. Peak Purity plot of sample: (A) Control, (B) Acidic Degradation, and (C) Alkaline Degradation.

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In the stability-indicating RP-HPLC method, the peak purity plot is crucial for confirming that the method can successfully separate the parent drugs (MET and REM) from their degradation products. In controlled conditions, the peak purity for both drugs will be near unity, while under acidic and alkaline degradation, a decrease in peak purity reflects the formation of degradation products. This analysis ensures the method is stability-indicating and capable of identifying the drugs' degradation behavior under stress conditions.

Characterization of the Degradant Products by LC-MS

MS spectrum of MET and REM provides critical insight into the molecular structures and fragmentation patterns of these compounds, helping to confirm their identity and molecular weights. For MET, with a chemical formula of $C_4H_{11}N_5$•HCl and a molecular weight of approximately 165.63 g/mol, the MS spectrum typically shows a molecular ion peak at m/z 130, corresponding to the protonated form of MET $[M + H]^+$. This peak represents the molecule without the HCl salt, which is commonly lost during ionization in mass spectrometry. The fragmentation pattern reveals additional key peaks, such as m/z 71, which is indicative of the cleavage of the biguanide group, resulting in the loss of part of the molecular structure. Another significant fragment appears at m/z 60, reflecting the loss of methyl groups. These fragmentation peaks help confirm the identity of MET by matching known degradation products and expected breakdown patterns during the ionization process.

In contrast, the MS spectrum of REM is more complex due to its larger molecular structure (**Figure 7**). With the chemical formula $C_{24}H_{32}O_8$ and a molecular weight of 448.51 g/mol, REM shows a molecular ion peak at m/z 449, representing the protonated form of the molecule $[M + H]^+$. This peak signifies the full molecular ion, including the etabonate group. Upon fragmentation, a key peak appears at m/z 329, which corresponds to the loss of the etabonate moiety ($C_5H_{10}O_3$), a protective ester group that is cleaved during the ionization process. Another significant fragment peak is observed at m/z 211, indicating the breakdown of the glycosidic bond, which is a crucial part of the glucose-like structure within REM. These fragmentation patterns allow for a deeper understanding of the structural components of REM, especially its sugar moiety and ester protection, confirming its identity and integrity during MS analysis. MS spectrum of MET showed a simpler structure with characteristic fragmentation ions that reflect its biguanide backbone and methyl groups. On the other hand, REM displays a more complex fragmentation pattern, with key losses of the etabonate and glycoside portions of the molecule. These spectral analyses provide valuable information for confirming the structure and purity of both MET and REM in pharmaceutical formulations.

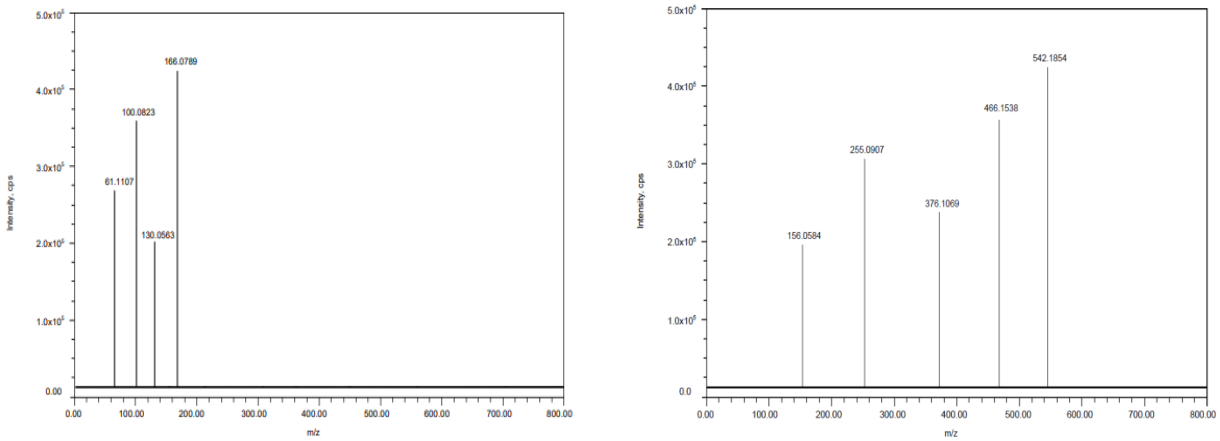

Figure 7. MS Spectrum of (A) Metformin HCl (B) Remogliflozin etabonate.

MS spectrum of MET and REM under forced degradation conditions reveals how these drugs degrade and what specific breakdown products are formed. This analysis is crucial in stability studies to determine the resilience of the drugs under various stress conditions, including acidic, alkaline, oxidative (peroxide), reductive, and thermal environments. Under acidic degradation conditions (**Figure 8**), such as exposure to hydrochloric acid, MET is relatively stable, though some minor hydrolysis may occur. The MS spectrum would still prominently display the molecular ion peak at m/z 130 ($[M+H]^+$), representing the intact MET molecule. However, minor fragment peaks could emerge, such as at m/z 71 and m/z 60, indicating some breakdown of the biguanide structure. REM, on the other hand, is more susceptible to acidic hydrolysis, particularly in its glycosidic and ester bonds. The molecular ion peak at m/z 449 ($[M+H]^+$) would decrease in intensity, with prominent fragment peaks appearing at m/z 329 (indicating the loss of the etabonate group) and m/z 211 (showing cleavage of the sugar moiety). Additional peaks might appear, representing smaller degradation products formed during the acid treatment.

In alkaline degradation conditions (**Table 8**), both compounds undergo more significant breakdown. MET, being a basic compound itself, is more resistant to alkaline conditions, but prolonged exposure can cause some deamination or

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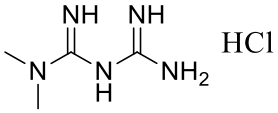
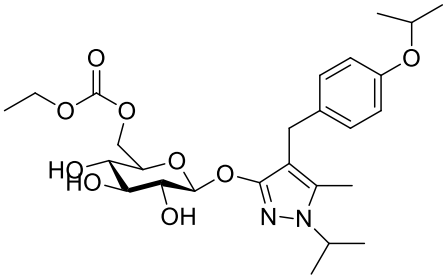
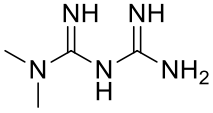
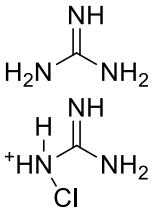
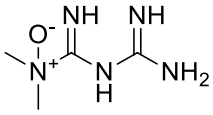
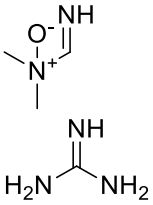
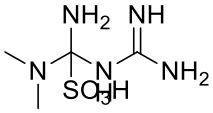
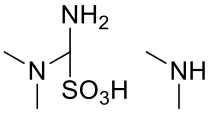
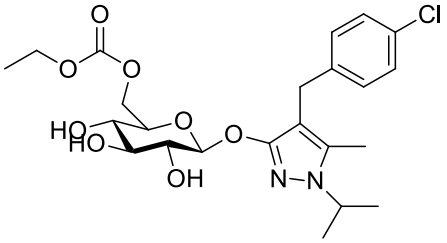
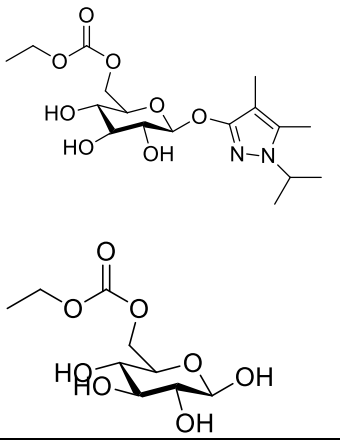
cleavage of the methyl groups. The MS spectrum may still show the m/z 130 peak for intact MET, but smaller fragments like m/z 60 (from methyl group loss) could become more pronounced. REM, however, is highly sensitive to alkaline hydrolysis. The molecular ion peak at m/z 449 would diminish, with the m/z 329 peak from etabonate cleavage and m/z 211 from glycosidic bond cleavage being more dominant. The degradation products from alkaline treatment would likely cause the formation of multiple smaller peaks in the MS spectrum, indicating the extensive breakdown of the parent molecule.

Table 8. Degradant products by LC-MS.

S. No.	Name	Precursor Ion (m/z)	Daughter Ion (m/z)	Chemical Structure (P)	Chemical Structure (D)
1	MET	165.0781	-		-
2	REM	522.2577	-		-
3	MET acid impurity (DP-1)	130.1022	60.0487 and 95.0173		
4	MET Peroxide impurity (DP-2)	146.0969	60.0492 and 89.0642		
5	MET Reduction impurity (DP-3)	211.0739	46.0582 and 155.0419		
6	REM acid impurity (DP-4)	489.1851	105.0477, 151.0536, 253.0849, 389.1851, and 499.1778		

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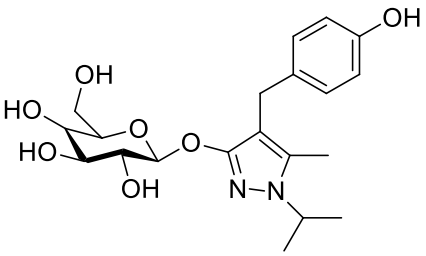
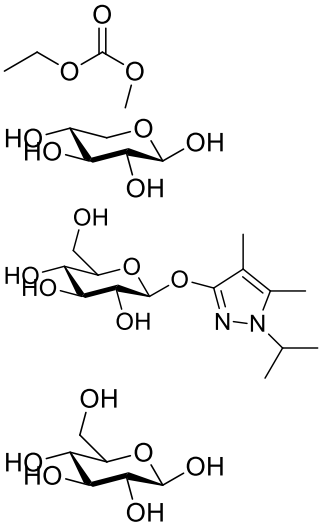
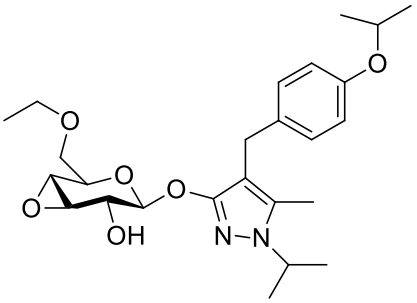
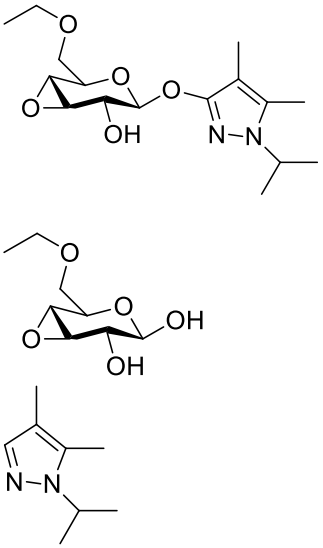
7	REM Peroxide impurity (DP-5)	409.1905	317.1645 and 181.0641		
8	REM Thermal impurity (DP-6)	461.2578	139.1165, 191.0847, and 327.1849		

In the case of peroxide degradation, oxidative stress induces the formation of reactive oxygen species that can attack both MET and REM. MET is generally stable under oxidative conditions, with the m/z 130 peak still evident, though some oxidation of the nitrogen groups may lead to additional peaks around m/z 140 due to the formation of oxidized MET derivatives. REM is more prone to oxidation, and the peroxide treatment can break down its sensitive ester and glycosidic bonds, leading to fragmentation. The MS spectrum would show a significant decrease in the m/z 449 peak, with an increase in degradation peaks at m/z 329 and m/z 211. Oxidized degradation products might also appear at higher masses, reflecting the addition of oxygen atoms to the structure. Under reductive degradation conditions, the MS spectrum changes as reduction targets specific chemical bonds in both compounds. For MET, reduction could lead to the saturation of nitrogen groups, though the m/z 130 peak is likely to remain dominant, as the molecule is relatively small and stable. However, some reduced fragments may appear at lower masses due to the cleavage of its structure. REM, being a more complex molecule, is more susceptible to reduction, particularly in its ester bonds and sugar moieties. The MS spectrum would show a decrease in the m/z 449 peak, with the formation of reduced fragments at m/z 329 and m/z 211, indicating significant structural changes. Additional lower mass peaks could emerge, reflecting the breakdown of these components under reductive conditions.

Lastly, thermal degradation can induce both structural rearrangement and decomposition. MET is thermally stable, and its MS spectrum under heat exposure would still show the m/z 130 peak, though prolonged heating might lead to some fragmentation, with peaks like m/z 71 and m/z 60 becoming more prominent. REM is more thermally sensitive, and its ester and glycoside bonds are likely to break under heat stress. The MS spectrum would show a significant reduction in the m/z 449 peak, with the degradation products appearing as prominent peaks at m/z 329 and m/z 211. Additional fragmentation due to thermal stress could produce even smaller degradation products, with corresponding peaks in the lower mass range. The MS spectra under forced degradation conditions reveal the stability profiles of MET and REM. MET shows relative stability under most conditions, with minor fragmentation occurring under acid, alkaline, and thermal degradation. REM, being more complex, is highly susceptible to degradation, particularly under acidic, alkaline,

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and oxidative conditions, as evidenced by the prominent fragmentation peaks and the loss of the molecular ion peak. These findings are crucial for understanding the degradation pathways and ensuring the robustness of stability-indicating methods.

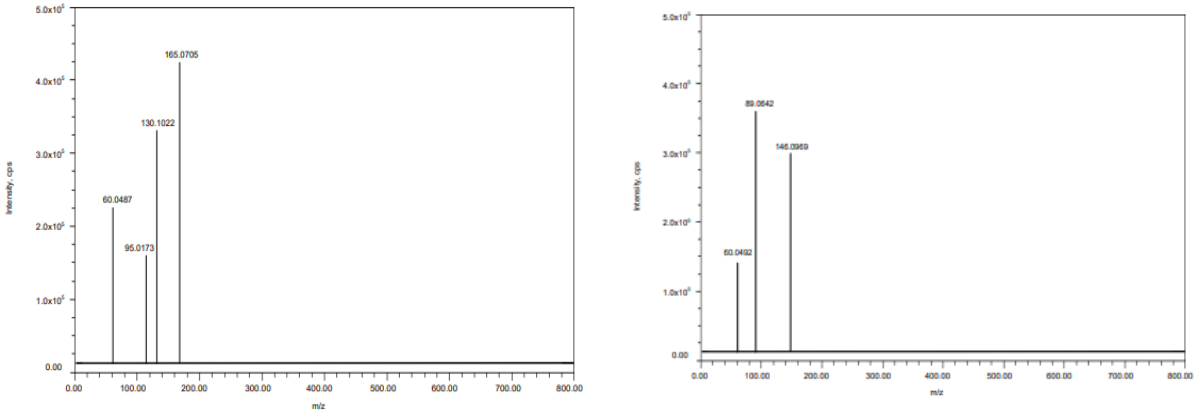

Figure 8. MS Spectrum of Metformin HCl DP-1 and DP-2 in (A) Acidic condition (B) Peroxide condition.

CONCLUSION

The study successfully developed and validated a robust stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous quantification of MET and REM in both pure and pharmaceutical dosage forms. The method proved to be highly specific, allowing for the effective separation of the two drugs and their degradation products under various stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic environments. The method demonstrated excellent linearity, precision, and accuracy, adhering to the stringent requirements outlined by ICH guidelines. The forced degradation studies provided critical insights into the stability profiles of MET and REM, revealing significant stability under neutral and oxidative conditions while highlighting their susceptibility to degradation under acidic, alkaline, and photolytic stress. This information is vital for understanding the degradation pathways and ensuring the quality and safety of the pharmaceutical products over their shelf life. Moreover, the method's robustness and reliability make it a valuable tool for routine quality control and stability testing in both industrial and research laboratories. The developed RP-HPLC method is not only suitable for the simultaneous estimation of these two drugs in their pure form but also in complex pharmaceutical formulations, ensuring the integrity and efficacy of the dosage forms throughout their lifecycle. This work contributes significantly to the field of pharmaceutical analysis, offering a comprehensive solution for the stability testing of MET and REM, thereby supporting the ongoing efforts to ensure the delivery of safe and effective pharmaceutical products to patients.

Conflict of Interest

Declared none

Funding information

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Animal ethics

Not required

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