

## Development and Validation of Stability indicating liquid chromatography method for the simultaneous quantification of Remogliflozin Etabonate, Vildagliptin and Metformin in combined formulation dosage forms

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### Abstract:

The fixed dose combination of remogliflozin etabonate, vildagliptin and metformin was recently approved for the treatment of type 2 diabetes and can improve glycemic control in patients. In the literature, there is no HPLC method reported for the estimation of remogliflozin etabonate, vildagliptin and metformin and hence the present study was aimed to develop a simple stability indicating HPLC method for the quantification of these analytes in combined dosage forms. The optimized and best separation was achieved using Phenomenex Luna C18 (250 mm × 4.6 mm, 5 µm) as stationary phase, pH 5.2 acetate buffer and acetonitrile in 55:45 (v/v) as mobile phase at 1.0 mL/min and PDA detection at 218 nm. The method reported a sensitive detection limit of 0.375 µg/mL, 1.875 µg/mL and 0.188 µg/mL with linearity range of 5-30 µg/mL, 25-150 µg/mL and 2.5-15 µg/mL respectively for remogliflozin, vildagliptin and metformin. The method passes all the validation parameters as per the guidelines proved that the method was valid. The method can shows very less % degradation in various stress studies such as acidic, base, peroxide, thermal and UV light

conditions and can effectively separate various stress degradation compounds confirms the stability indicating nature of the method. The method can adequately suitable for the separation and quantification remogliflozin, vildagliptin and metformin in formulation hence can be applicable for the routine analysis of remogliflozin, vildagliptin and metformin in pharmaceutical dosage forms.

**Key words:** Remogliflozin etabonate, Vildagliptin and Metformin, HPLC analysis, Forced degradation studies

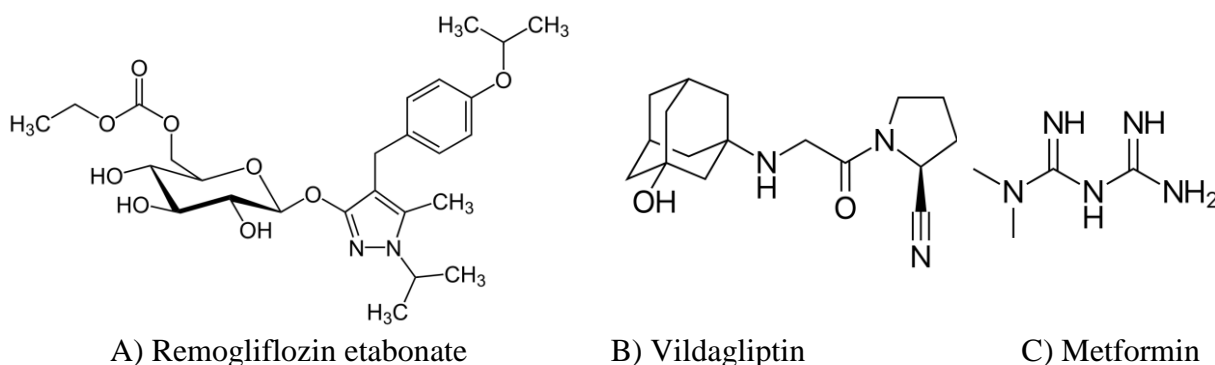
### Introduction:

Remogliflozin etabonate [Figure 1A] is a gliflozin class anti-diabetic drug prescribed for the treatment of non-alcoholic steatohepatitis and type II diabetes [1]. In type 2 diabetes patients remogliflozin etabonate reduces glucose concentrations in plasma [2]. Remogliflozin works by inhibiting the SGLT protein that was responsible for the reabsorption of glucose in kidney. The results of blockage of SLGT protein leads to the elimination of blood glucose through urine. In a health subjects, a single dose of 1000 mg increase the glucose excretion through urine than the 500 mg and hence the drug is well tolerated with subjects to II diabetes mellitus. The possible side effects of remogliflozin include genital mycotic infections, dizziness and urinary tract infections [3].

Vildagliptin [Figure 1B] is a cyanopyrrolidine class first gliptin drug used in the treatment of type II diabetes mellitus [4]. Vildagliptin decreases the blood glucose levels by inhibiting dipeptidyl peptidase-4. Tremors, nausea, dizziness, headache and hypoglycaemia are the side effects of vildagliptin. In some rare cases hepatotoxicity was also identified in some patients while using vildagliptin [5]. It was available in single or in combination with other drugs such as metformin and remogliflozin etabonate.

Metformin [Figure 1C] is the first-line medication prescribed for treating type 2 diabetes especially for the patients who are overweight [6]. It was also used in treating polycystic ovary syndrome that was not associated with weight gain [7]. It was the most widely prescribed medication for treating diabetes with oral administration and was included in list of essential medicine by WHO. The possible side effects associated with the use of metformin are the gastrointestinal irritation includes cramps, vomiting, increased flatulence, nausea and diarrhea [8].

The literature survey for the available analytical methods confirms that only one hydrophilic interaction liquid chromatography method was reported for the analysis of remogliflozin etabonate, vildagliptin and metformin [9]. One LCMS method was reported for the simultaneous quantification of remogliflozin etabonate and vildagliptin in biological samples [10]. Few methods reported for the simultaneous quantification of metformin in combination with remogliflozin etabonate [11-13] and vildagliptin [14-17] using various analytical techniques. Based on the literature review, it was identified that there is no simple and convenient analytical method was available for the simultaneous quantification of remogliflozin etabonate, vildagliptin and metformin. Hence the present work was intended to develop a simple and convenient HPLC method for the separation and quantification of remogliflozin etabonate, vildagliptin and metformin in pharmaceutical formulations. The molecular structures of analytes in the study was given in figure 1.



**Figure 1: Molecular structure of analytes in the study**

## Materials and Methods:

### Reagents:

The API of remogliflozin etabonate, vildagliptin and metformin were procured from Glenmark Pharmaceuticals Ltd, Hyderabad. The formulation tablets with brand Remo-Zen MV<sup>®</sup> tablets with dosage of 100 mg, 50 mg and 500 mg respectively for remogliflozin etabonate, vildagliptin and metformin were purchased from local pharmacy. The ultra-pure (Milli-Q<sup>®</sup>) water and other HPLC grade solvents used in the study were purchased from Merck chemicals, Mumbai. The analytical reagent grade chemicals used in the study such as hydrochloric acid, sodium hydroxide, hydrogen peroxide, and buffer chemicals were also purchased from Merck chemicals, Mumbai.

### Instrumentation:

The study was conducted on Agilent (USA) 1100 HPLC instrument that comprises of G1311 A quaternary pump for delivery of solvents, 0.1 – 1500  $\mu$ L volume injectable auto-sampler with thermostat and PDA detector. Various configurations of stationary phases were used for the method development studies and the column eluents were integrated using Agilent chemstation software.

#### **Preparation of standard stock solutions:**

An accurately weighed 50 mg of analytes in the study such as remogliflozin etabonate, vildagliptin and metformin were dissolved separately in a 50 mL clean and dry volumetric flask. Then 25 mL of methanol was added separately in each flask and sonicate the flasks for 2 min to dissolve the analytes completely in the solvent. Then the content was filtered through 0.2  $\mu$  membrane filter in a separate clean and dry flask separately and the final volume was made up to the mark with the same solvent. The standard remogliflozin etabonate, vildagliptin and metformin solution at a concentration of 1000  $\mu$ g/mL was obtained separately. The mixed standard solution containing all the analytes was prepared by mixing equal volumes of known and selected concentration of analytes in a separate clean and dry 10 mL volumetric flask. The mixed standard solutions containing selected known concentrations of remogliflozin etabonate, vildagliptin and metformin was used for method development and validation study.

#### **Preparation of formulation solution:**

The formulation tablets with brand Remo-Zen MV<sup>®</sup> were made in to a fine and uniform powder using a clean and dry mortar and pestle. The tablet powder weighed such that the weighed powder contain 10 mg equivalent of remogliflozin etabonate was taken in a clean and dry 100 mL volumetric flask. Then 50 mL of diluent was added and sonicated for 10 min at room temperature. Then the content was filtered through 0.2  $\mu$  membrane filter in a separate clean and dry flask and the volume was made up to the mark using the same solvent. The filtered solution was diluted such that the solution contains 15  $\mu$ g/mL of remogliflozin etabonate and based on the formulation dosage, the solution contains 75  $\mu$ g/mL of metformin and 7.5  $\mu$ g/mL of vildagliptin. The solution was used for the formulation assay of the analytes in the developed method.

#### **Method development:**

The development of method for the separation and simultaneous quantification of remogliflozin etabonate, vildagliptin and metformin was carried by systematic method development strategies. The PDA detector wavelength was confirmed by measuring the

maximum absorption wavelength in the detector. The obtained maximum absorbing wavelength was selected as suitable wavelength for the detector. In the initial method development steps, the mobile phase flow rate was fixed as 1.0 mL/min and after the completion of the development; the flow was further optimized in the range of 0.5 mL/min to 1.5 mL/min. The analytes in the study were polar in nature and the non-polar columns were utilized as stationary phases in the development of method. The high non-polar c18 columns of various brands and configurations were studied as stationary phase in the development study. The composition and pH of the mobile phase was finalized by change in various compositions of the mobile phase with different pH ranges was studied. In the development of analytical method, the instrument was stabilized with selected method conditions and the standard solution containing 15 µg/mL of remogliflozin etabonate, 7.5 µg/mL of vildagliptin and 75 µg/mL of metformin was injected. The peak area response, peak intensity, peak shape and the system suitability was summarized in all the studied conditions. The method conditions that produce best system suitability with high peak intensity and significantly no noise was considered as suitable conditions for the separation and analysis of remogliflozin etabonate, vildagliptin and metformin [18-21]. These developed method conditions were further studied for method validation study.

#### **Method Validation:**

The standard solution containing 15 µg/mL of remogliflozin etabonate, 7.5 µg/mL of vildagliptin and 75 µg/mL of metformin was analysed in the optimized method and the chromatographic response of the resultant chromatograms was summarized for the evaluation of the system suitability of the developed method. The diluent with no analyte was considered as blank and placebo (solution prepared with commonly used formulation excipients) analysis was also analysed in the developed method for the evaluation of the excipients interference in the developed method.

The formulation dosage was considered for preparing the linear calibration curve dilutions. Various concentration ranges were prepared and the prepared dilutions was analysed in the developed method and the peak area response of analytes were tabulated separately. The calibration curve was constructed for the three analytes separately by taking the peak area response of individual analyte in y-axis and its concentration on x-axis. The correlation coefficient and the regression equation was derived from its corresponding calibration graphs. The accuracy of the method established for the analysis of remogliflozin etabonate,

vildagliptin and metformin was evaluated by performing the spiked recovery study at 50%, 100% and 150% spiked levels. The spiked level solution of remogliflozin etabonate, vildagliptin and metformin was spiked to a 100 % formulation solution and the recovery solution was analysed in the optimized method. The peak area response of the recovery solution was compared with the calibration curve results in the same level and the % recovery of each analysis results and the % relative standard deviation (% RSD) in each spiked level was calculated. The % recovery in the level of 98-102 and the %RSD of less than 2 was considered as acceptable.

The reproducibility of the method was evaluated in terms of precision and was carried as intraday and interday precision. In this, the standard solution containing 15 µg/mL of remogliflozin etabonate, 7.5 µg/mL of vildagliptin and 75 µg/mL of metformin was assessed six times in one day for intraday precision and 6 times in three consecutive days for interday precision. The peak area response of individual analyte was tabulated and the %RSD of the peak area response was calculated. The %RSD of less than 2 in both the precision studies for all the analytes was considered as the method was precise and repeatable.

In ruggedness study, the solution at precision level was prepared and analysed by three different analysts and the % RSD of the peak area response was calculated and the %RSD of less than 2 was considered as the method was rugged. In robustness study, both positive and negative minor variations in the established method conditions made intentionally and the standard solution at precision level was analysed in each changed condition. The % change in the peak area response of standard and its impurities was calculated and a % change of less than 2 was considered as the method was robust. The minimum analyte concentration that can detect and quantify the analytes in the developed method was considered as limit of detection and quantification respectively. This information of the developed method confirms its sensitivity. The signal (s) to noise (n) ratio method was adopted for the evaluation of sensitivity.

The stability indicating nature of the method was assessed by performing stress degradation studies and the stress studies such as acidic, base, peroxide, thermal and UV light degradation studies was performed to the standard drug. The standard solution (50 mL) was mixed with 50 mL of hydrochloric acid (0.1 N), sodium hydroxide (0.0 N) and hydrogen peroxide (3 %) in acid, base and peroxide degradation studies respectively. The solution was kept in dark for

24 h, neutralized using hydrochloric acid/sodium hydroxide solution. In thermal degradation, the standard drug was kept in an air oven at 60 °C for 24 h whereas in UV light degradation study, the standard drug was kept under UV light for 24 h. After exposing the stress condition, the standard drug was diluted to standard concentration and was analysed in the developed method. The resultant chromatograms confirms the number of degradation compounds formed during the stress exposure of standard drug and the effectiveness of the method for the separation of degradation compounds was confirmed. The peak area response observed in each study was used for calculating the % degradation of remogliflozin etabonate, vildagliptin and metformin by comparing with un-stressed peak area response in the developed method [18-21].

The developed method was applied for the separation, detection and quantification of remogliflozin etabonate, vildagliptin and metformin in formulation. The formulation sample solution prepared from Remo-Zen MV<sup>®</sup> tablets was analysed in the developed method. The peak area response was used to calculate the % content in the sample by comparing with corresponding standard calibration curve results.

### Results and Discussions:

The detector wavelength of the analytes was confirmed by scanning in PDA detector and based on the obtained absorption spectra, it was observed that 218 nm wavelength was confirmed as suitable for the detection of analytes. In the initial method development, 100 mm columns were studied as stationary phase. In the initial development, acetate buffer pH 5.6 and methanol in the ratio of 75:25 (v/v) as mobile phase at 1.0 mL/min flow rate. The Inertsil ODS C18 (100 mm × 4.6 mm, 5 µm) column, ProntoSIL ODS C18 (100×4.6 mm; 5 µ) column and Phenomenex Luna C18 (100 mm × 4.6 mm, 5 µm) column were studied as stationary phase separately. There is no clear separation of analytes was observed while using Inertsil C18 (Figure 2A) and ProntoSIL C18 columns (Figure 2B) as stationary phases confirms that these column were not able to separate the analytes in the study whereas the chromatogram observed for Phenomenex Luna C18 column (Figure 3C) shows clear separation of analytes proved that the column was suitable for the separation of analytes but the resolution between the identified compounds was observed to be very poor and hence further trials were continued with 250 mm column with change in the composition of the mobile phase. In continuation of the method development, Phenomenex Luna C18 (250 mm × 4.6 mm, 5 µm) as stationary phase and pH 5.2 acetate buffer and acetonitrile in the ratio of

25:75 (v/v) was studied as mobile phase at 1.0 mL/min. In these conditions, clear separation of analytes was identified with acceptable resolution. The peak corresponding to remogliflozin etabonate was observed to be little broad with high tail factor and poor theoretical plates (Figure 3D). Figure 3 represents the chromatograms observed in the method development study.

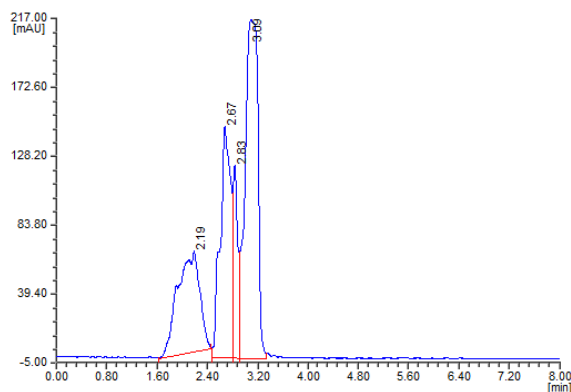


Figure 2A

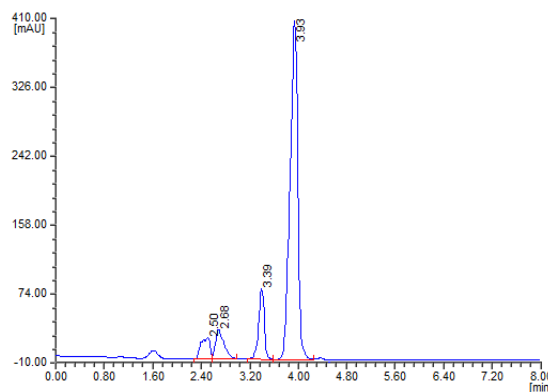


Figure 2B

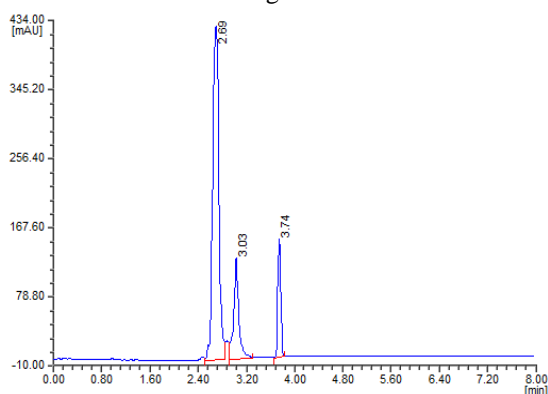


Figure 2C

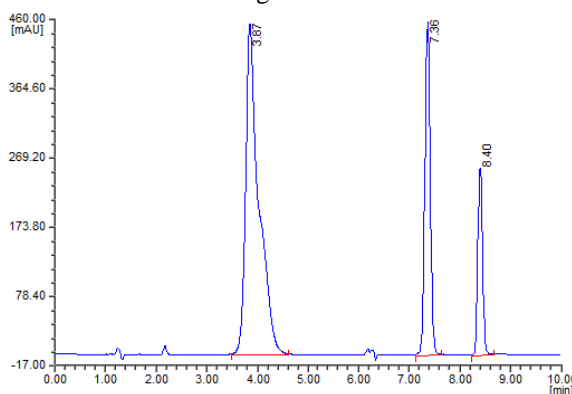


Figure 2D

### Figure 2: Method development trail chromatograms

The method development trails was continued with increase and decrease in the composition of buffer in mobile phase, flow rate of mobile phase and detector wavelength for achieving the best chromatographic separation with acceptable system suitability. The separation was achieved on Phenomenex Luna C18 (250 mm × 4.6 mm, 5 μm) as stationary phase, pH 5.2 acetate buffer and acetonitrile in 55:45 (v/v) as mobile phase at 1.0 mL/min and PDA detection at 218 nm. In these optimized chromatographic conditions, clear separation of remogliflozin etabonate, vildagliptin and metformin was achieved with no additional detection of impurities or other co-eluting compounds. The analytes were identified at a retention time of at a retention time of 3.40 min for remogliflozin etabonate, 7.38 min for



metformin and 8.41 min for vildagliptin whereas the chromatogram of blank doesn't show any chromatographic detections throughout the run time. This confirms that the established method was specific for the detection of remogliflozin etabonate, vildagliptin and metformin. The chromatogram of blank and standard observed in the developed method condition was represented in figure 3A and 3B respectively.

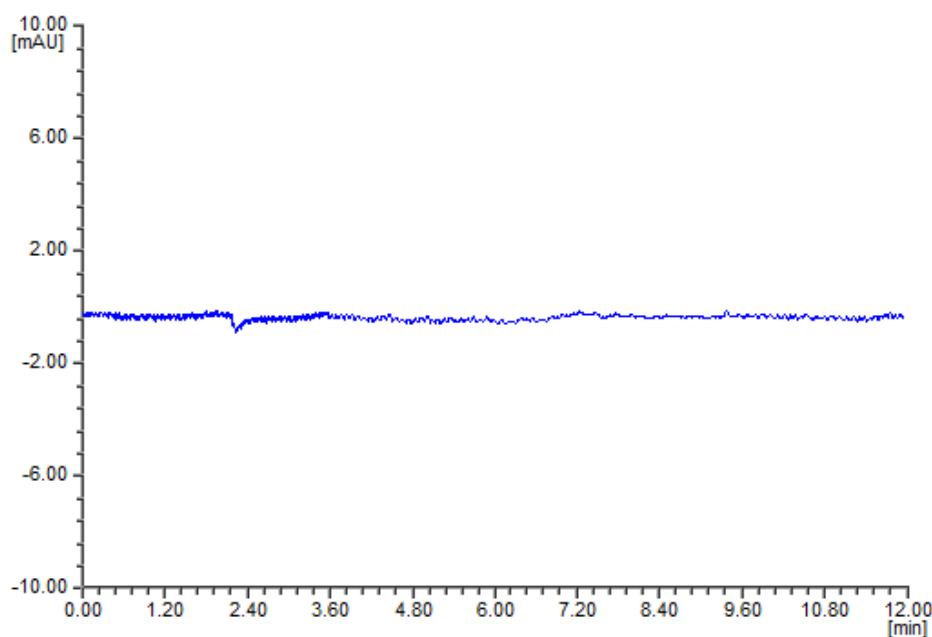


Figure 3A (Blank chromatogram)

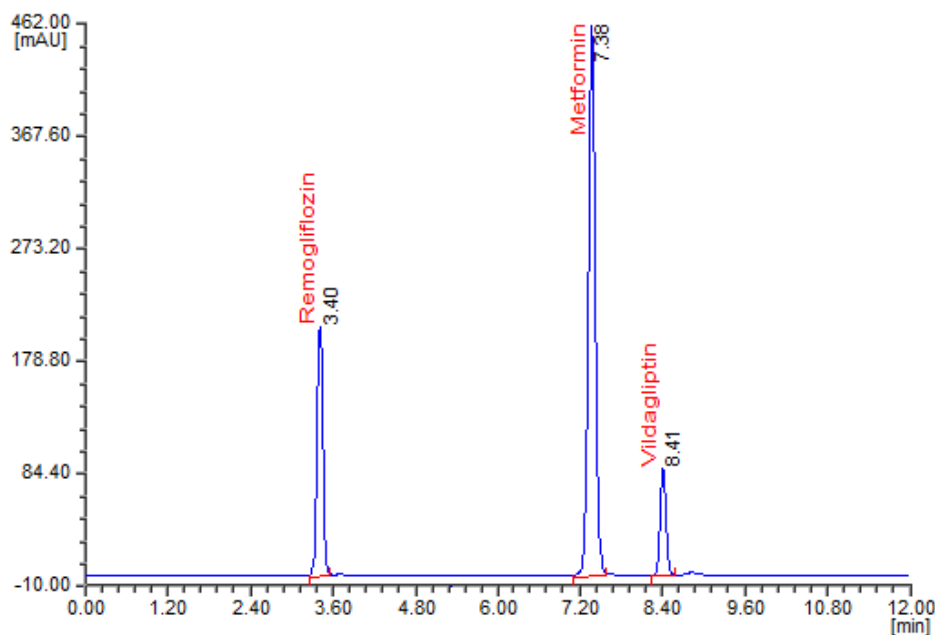


Figure 3B (Standard chromatogram)

**Figure 3: System suitability chromatograms in the developed method**

The sensitivity of the developed method was assessed in terms of detection (LOD) and quantification (LOQ) limits and was evaluated using s/n approach. The LOD was identified as 0.375 µg/mL for remogliflozin etabonate, 1.875 µg/mL for metformin and 0.188 µg/mL for vildagliptin whereas the LOQ was calculated as 1.238 µg/mL, 6.188 µg/mL and 0.619 µg/mL for remogliflozin etabonate, metformin and vildagliptin respectively. This confirms that the method can effectively detect the analytes up to low concentrations for all the analytes and based on the sensitivity levels it was confirms that the method was very sensitive.

A six point linear calibration curve was observed with in the concentration levels of 5-30 µg/mL for remogliflozin etabonate, 25-150 µg/mL for metformin and 2.5-15 µg/mL for vildagliptin. The regression equation derived as  $y = 8488.1x + 34321$  ( $R^2 = 0.9992$ ),  $y = 2992.9x + 44911$  ( $R^2 = 0.9993$ ) and  $y = 6314.1x + 16703$  ( $R^2 = 0.9992$ ) respectively for remogliflozin etabonate, metformin and vildagliptin. The peak area response obtained in the linearity study was represented in table 1.

S. No	Remogliflozin		Metformin		Vildagliptin	
	Concentration in µg/mL	Peak Area	Concentration in µg/mL	Peak Area	Concentration in µg/mL	Peak Area
1	5	77042.6	25	116471.9	2.5	33328.7
2	10	121392.5	50	198424.2	5	48234.1
3	15	157473.6	75	269623.4	7.5	63490.3
4	20	204584.2	100	341632.8	10	78591.4
5	25	247848.9	125	424251.6	12.5	95791.7
6	30	288829.1	150	490328.0	15	112269.9

**Table 1: Linearity results**

The standard solution at a concentration of 15 µg/mL of remogliflozin etabonate, 7.5 µg/mL of vildagliptin and 75 µg/mL of metformin was analysed in the developed method for the evaluation of system suitability. The system suitability parameters of the chromatographic results were summarized and the method system suitability was assessed. As summarized in table 2, the developed method passes the system suitability confirms the suitability of the developed method.

S No	Parameter	Results achieved for		
		Remogliflozin	Metformin	Vildagliptin
1	Concentration prepared	15 µg/mL	7.5 µg/mL	75 µg/mL

2	Retention Time	3.40 min	7.38 min	8.41 min
3	Theo plate	4271	5814	9428
4	Tail Factor	1.04	0.97	0.99
5	Resolution	--	9.58	4.90

**Table 2: System suitability results**

The standard solution containing 15 µg/mL of remogliflozin etabonate, 7.5 µg/mL of vildagliptin and 75 µg/mL of metformin was used for the evaluation of repeatability and reproducibility of the developed method. The peak area response of each analyte was summarized in each study and the % RSD was calculated as 0.63, 0.66 and 0.50 in intraday precision, 0.71, 0.73 and 0.60 in interday precision and 0.77, 0.89 and 0.74 in ruggedness for remogliflozin etabonate, metformin and vildagliptin respectively. The % RSD results falls under the acceptable levels for all the analytes in each study proved that the method was precise and rugged.

The impact of the minor variations in the developed method conditions on the chromatographic response of remogliflozin etabonate, metformin and vildagliptin was assessed in robustness study. In this, the composition of mobile phase was varied as 50:50 (MP 1) and 60:40 (MP 2) of buffer and methanol. The pH of buffer was altered as 5.1 (pH 1) and 5.3 (pH 2) as well as the detector wavelength of changed as 213 nm (WL 1) and 223 nm (WL 2). In all the changed varied conditions, the % change in the peak area response of the analytes was calculated and found that the % change was under the acceptable limit of less than 2 for all the analytes (Table 3). There is no considerable variation in the peak shape and system suitability of the analytes proved that the method was robust.

S No	Change	Remogliflozin		Metformin		Vildagliptin	
		Peak Area	% Change	Peak Area	% Change	Peak Area	% Change
1	MP 1	156371.1	0.70	266443.7	1.18	63726.3	0.37
2	MP 2	156641.7	0.53	266910.0	1.01	62952.7	0.85
3	pH 1	157332.8	0.09	264709.5	1.82	62895.2	0.94
4	pH 2	159395.1	1.22	267167.4	0.91	63711.0	0.35
5	WL 1	156547.1	0.59	265595.5	1.49	63373.8	0.18
6	WL 2	157349.3	0.08	266521.8	1.15	62913.4	0.91

**Table 3: Robustness results**

The spiked recovery at 50%, 100% and 150% spiked levels was performed for the evaluation of the accuracy of the developed method. The spiked level solution was analysed in the developed method and the peak area response of the analytes was compared with its calibration results in the same concentration level and the % recovery was calculated for each analysis. The % RSD of the recovery in each spiked level for the three analytes was calculated and the results were tabulated in table 4. The % recovery was achieved under the acceptable level of 98-102 % for the three analytes in each analysis and the % RSD was observed to be under the acceptable level of less than for the three analytes in each studied spiked level confirms that the method was accurate.

S. No.	Compound	Recovery Level	Concentration in $\mu\text{g/mL}$			Amount found* Mean $\pm$ SD	% recovered* Mean $\pm$ SD	% RSD of Recovery
			Target	Spiked	Final			
1	Remogliflozin	50 %	10	5	15	14.88 $\pm$ 0.011	99.22 $\pm$ 0.076	0.08
2		100 %	10	10	20	19.74 $\pm$ 0.114	98.69 $\pm$ 0.570	0.58
3		150 %	10	15	25	24.76 $\pm$ 0.200	99.03 $\pm$ 0.800	0.81
4	Metformin	50 %	50	25	75	74.02 $\pm$ 0.188	98.69 $\pm$ 0.251	0.25
5		100 %	50	50	100	98.88 $\pm$ 0.422	98.88 $\pm$ 0.422	0.43
6		150 %	50	75	125	123.84 $\pm$ 1.059	99.07 $\pm$ 0.847	0.86
7	Vildagliptin	50 %	5	2.5	7.5	7.40 $\pm$ 0.046	98.72 $\pm$ 0.611	0.62
8		100 %	5	5	10	9.94 $\pm$ 0.026	99.44 $\pm$ 0.263	0.26
9		150 %	5	7.5	12.5	12.32 $\pm$ 0.053	98.59 $\pm$ 0.425	0.43

\* n=3

**Table 4: Recovery results**

The method was studied evaluated for its applicability for the separation and analysis of various compounds generated due to stress degradation of remogliflozin etabonate, metformin and vildagliptin. The stress exposed solutions were analysed in the developed method and the resultant chromatograms were analysed for the evaluation of its applicability for the separation of stress degradants. In acid degradation study, 5 degradation compounds were separated and identified in the chromatogram. The % degradation was calculated as 8.12, 9.37 and 6.85 respectively for remogliflozin, metformin and vildagliptin. In base degradation the % degradation was calculated as 8.57, 5.85 and 7.52 for remogliflozin etabonate, metformin and vildagliptin respectively and the chromatogram clearly shows the separation of 4 degradation compounds. The degradation chromatograms of peroxide,

thermal and UV light shows the clear separation of 3, 4 and 4 degradation compounds and the % degradation was less compared with acidic stress studies. In all the stressed conditions performed, the standard remogliflozin etabonate, metformin and vildagliptin were detected along with the two impurities in the study. The % degradation was noticed to be less in all the stressed conditions and the method can effectively resolve the stress degradants effectively proved that the method was stable. The stress study chromatograms were shown in figure 4.

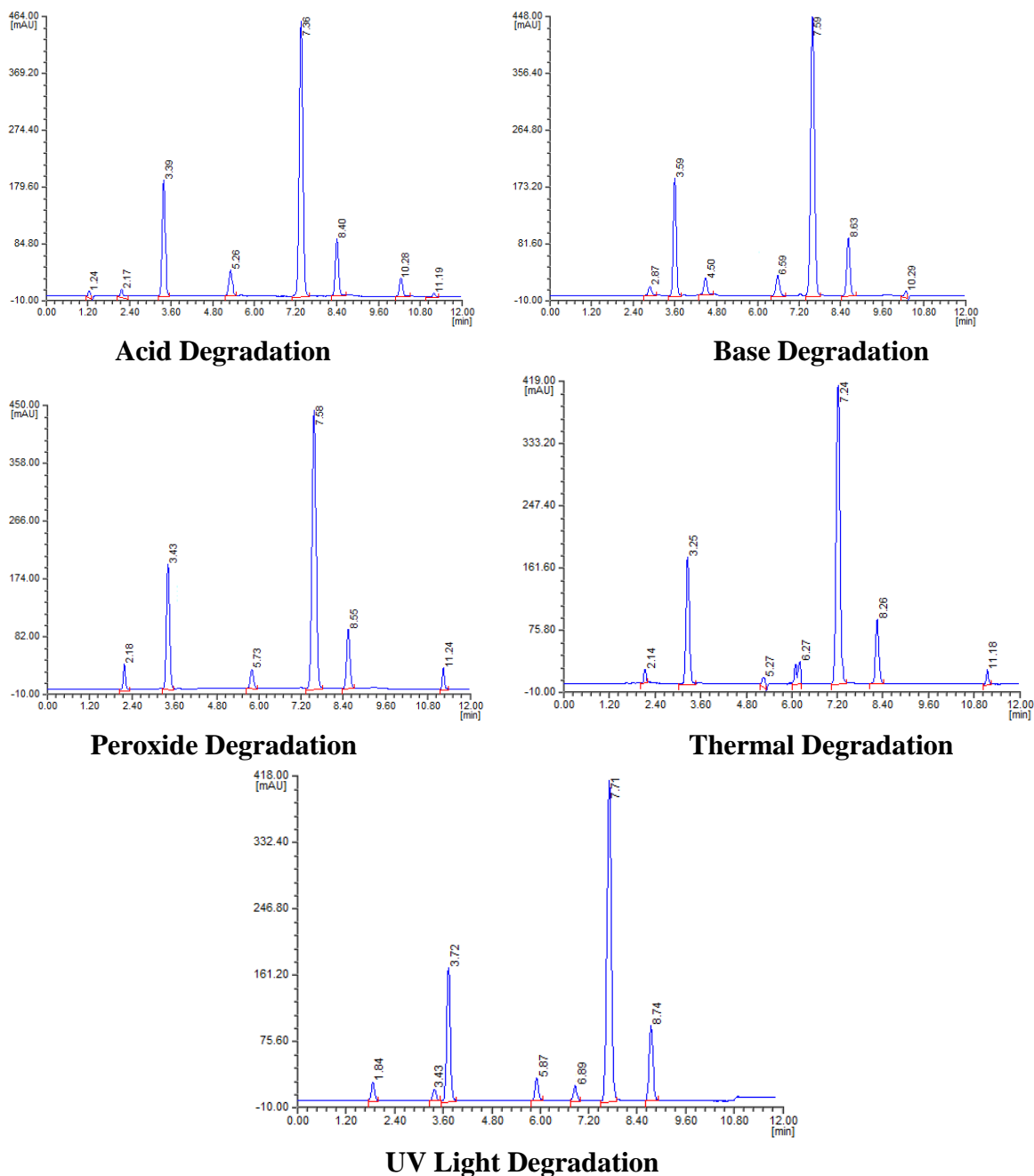
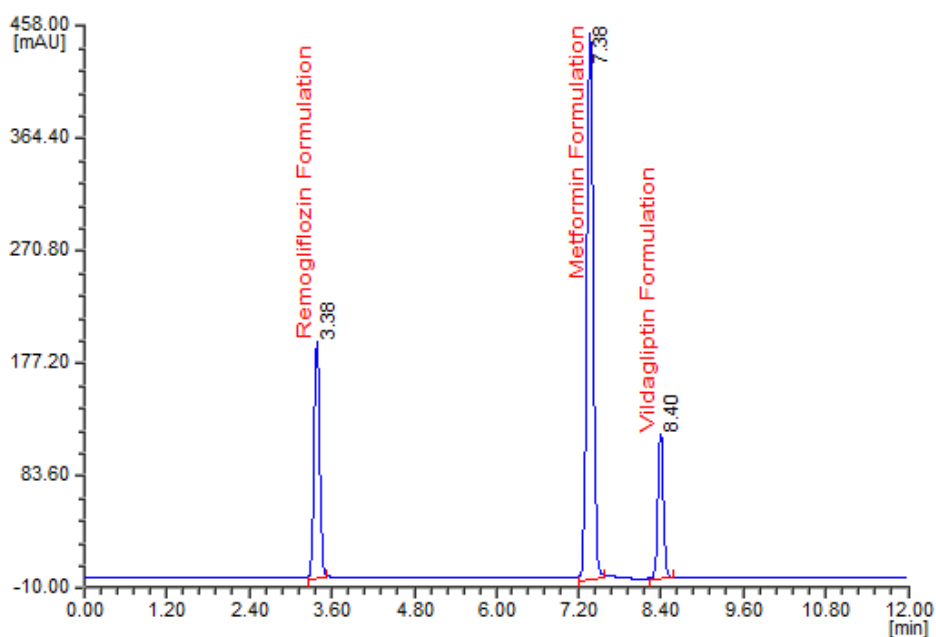


Figure 4: Stress degradation chromatograms in the developed method

The analytical method optimized in the study was applied for its applicability for the estimation of remogliflozin, metformin and vildagliptin in formulation. The formulation solution prepared using marketed tablets was used for the formulation assay study. The resultant chromatogram show clear identification and resolution of analytes in the study. The % assay was calculated as to be 98.34, 99.08 and 99.36 for remogliflozin, metformin and vildagliptin respectively. The % assay of more than 98 % was observed for all the analytes and the chromatogram doesn't show any additional detections. Hence the method can effectively utilized for the analysis of remogliflozin, metformin and vildagliptin.



**Figure 5: Formulation chromatogram**

### Conclusion:

A simple and accurate stability indicating analytical RP-HPLC method was optimized for the resolution, qualitative and quantitative evaluation of remogliflozin, metformin and vildagliptin in bulk drug and formulations. The method can effectively separate the analytes with no addition detection of impurities or the formulation excipients. The method reports the detection limit as 0.375  $\mu\text{g/mL}$  for remogliflozin etabonate, 1.875  $\mu\text{g/mL}$  for metformin and 0.188  $\mu\text{g/mL}$  for vildagliptin. The method passes the validation parameters such as specificity, system suitability, accuracy/recovery, repeatability and reproducibility. The developed method can effectively separate the unknown stress degradation products and the % degradation was reported to less in all the stressed conditions studied. Based on the achieved results, it can be concluded that the method developed in the study can effectively utilized for the routine analysis of remogliflozin, metformin and vildagliptin in dosage forms.

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