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Stability indicating liquid chromatographic method for the estimation of remogliflozin etabonate

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Abstract: Accurate and precise reverse phase liquid chromatographic method has been developed for the estimation Remogliflozin etabonate in bulk and tablet dosage form. Reverse phase C18 column was used as stationary phase along with mixture of methanol:water (70:30%, v/v) as a mobile phase. Mobile phase flow rate was maintained at 1mL/min and analysis was performed at 229 nm. The method was linear in the concentration range of 1 – 25 µg/mL with correlation coefficient (r^2) 0.997. The proposed method was validated with respect to linearity, accuracy, precision and robustness as per ICH Q2 (R1) guideline. To find out the possible degradation pathway, forced degradation studies were performed. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time value. The drug was found to be highly susceptible to acid and base hydrolysis. The developed method can be used for analysis of stability samples and routing quality control evaluation of Remogliflozin etabonate in tablet formulation.

Keywords: Remogliflozin etabonate; forced degradation; validation; liquid chromatography. © 2020 ACG Publications. All rights reserved.

1. 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. The molecular formula of REM is $C_{26}H_{38}N_2O_9$ and a molecular weight of 522.6 g/mol (Figure S1). This is an inactive prodrug which upon the administration and absorption is converted to its active form remogliflozin which acts particularly on the sodium-glucose co-transporter subtype 2 (SGLT2) and used for treatment of Diabetes Mellitus Type-2 [1-5].

A literature survey regarding quantitative analysis revealed that various analytical methods have been reported for the estimation of REM. Estimation of REM in human plasma has been reported by LC MS-MS [6-7] methods. UV Spectroscopy and HPTLC [8] have been developed for the analysis of REM in bulk and tablet dosage form. REM is not official in any pharmacopoeia.

Analytical method submitted to drug authority as a part of new drug application or abbreviated new drug application should be specific and it must have stability indicating nature. Stability studies help

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to identify the intrinsic stability of drug molecule and precautions to be observed while handling and manufacturing drug product. Liquid chromatography is the most acceptable method of analysis due to its accuracy, precision and sensitivity. No liquid chromatographic method has been reported for the estimation of REM, so the present study involves development of stability indicating liquid chromatographic method which has advantage of sensitivity, accuracy, rapidity, precision and specificity.

2. Experimental

2.1. Reagents and Material

Pure Sample and formulation- Analytically pure remogliflozin etabonate (99.12 %w/w) was obtained as gift samples from Glenmark Pharmaceutical Company, India. Marketed formulation with a brand name Remo-Zen (Each film coated tablet contains Remogliflozin etabonate 100 mg by Glenmark Pharmaceuticals Ltd., Mumbai, India) were procured from local market.

Methanol and water of HPLC grade were purchased Merck Ltd., Mumbai, India while sodium hydroxide, hydrochloric acid and 3% pydrogen peroxide of analytical reagent grade were purchased from S.D. Fine Chem Ltd. Mumbai, India.

2.2. Chromatographic Condition

HPLC System consisting of Infinity 1220 LC Agilent model along with Stationary phase containing Shimadzu ODS C18 column and mobile phase Methanol:Water (70:30%v/v) was used in the chromatographic study. The LC system was equilibrated with the mobile phase before starting analysis. The flow rate was maintained at 1 mL/min and eluent were monitored with diode array detector at 229 nm. Total run time was kept at 15 min.

2.3. Preparation of Stock Solution and Calibration Standards

REM (10 mg) was accurately weighed and transferred to 10 mL volumetric flask. 5 mL methanol was added and swirled to dissolve the drug. Volume was made up to the mark with methanol in order to prepare standard stock solution of 1000 µg/mL solution. 1 mL of this solution was withdrawn in 10 mL volumetric flask and volume was made to 10 mL with methanol to get working standard solution of 100 µg/mL.

From the above prepared stock solution, six different concentrations for REM was prepared with ranges from 1–25µg/mL. The appropriate amount of solution was withdrawn from the stock solution in 10mL volumetric flask and was further made up to the mark using methanol.

2.4. Validation

Validation of the proposed HPLC method was carried out according to International Conference on Harmonization (ICH) guidelines Q2 (R1) for linearity, accuracy, precision, repeatability, specificity, sensitivity, and robustness [9].

The linearity of method was analysed by preparing calibration curve at six concentration levels over the range of 1–25µg/mL. The calibration curve was established by plotting Peak area versus Concentration (n= 6) and straight line equation was find out.

The accuracy of the method was determined by calculating recoveries of REM by method of standard additions. Known amount of REM (50%, 100%, 150%) were added to a pre-quantified tablet formulation and the amount of REM was estimated by measuring the peak area and by fitting these values to the straight-line equation of calibration curve.

Intraday precision was determined by analyzing sample solutions of REM (1,10 and 25µg/mL) at three levels covering low, medium, and high concentrations of the calibration curve three times on the same day. Intraday precision was determined by analyzing sample solutions of REM (1,10 and 25µg/mL) at three levels covering low, medium, and high concentrations over a period of 3 days. The peak areas were obtained and %RSD values were determined. Repeatability of sample application was assessed by

analyzing REM (10 µg/mL) and six times and peak area was recorded. The percent relative standard deviation (RSD %) of mean peak areas were obtained.

Sensitivity of the method was determined with respect to LOD and LOQ. Noise was determined by scanning a blank injection of mobile phase six times. A series of concentrations of drug solutions 1 – 25µg/mL were injected and analyzed to determine LOD and LOQ. LOD was calculated as 3 times the noise level, and LOQ was calculated as 10 times the noise level.

The specificity of method was ascertained by analyzing REM in presence of excipients (Microcrystalline cellulose, Magnesium Stearate, Ethyl Cellulose, Methyl Paraben, Talc) which are used to prepare synthetic mixture [10-13]. The peak of REM was confirmed by comparing Rt value and chromatogram of standard. Robustness study was carried out by deliberate variations in the flow rate and mobile phase ratio. The study showed that deliberate changes in the mobile phase and flow rate showed no significant variation.

The system-suitability tests are integral part of gas and liquid chromatography. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The system suitability parameters like resolution, theoretical plate and asymmetric factor were calculated and compared with standard values. The system suitability test was carried out on freshly prepared working standard stock solution of 10µg/mL.

2.5. Forced Degradation Study

Forced degradation study help to identify the intrinsic stability of drug sample and also help to establish the degradation pathways. To perform forced degradation, REM was subjected to various stress conditions like acid - base hydrolysis, oxidative hydrolysis, thermal degradation and UV light exposure as mentioned in ICH Q1A (R2).

2.5.1. Alkali Hydrolysis

To perform alkali degradation study, appropriate aliquots of stock solutions 2 mL of (1000µg/mL) of REM was taken in 10 mL volumetric flasks and 2.5 mL of 0.1N NaOH was added then sample was heated for 30 mins and was further neutralized with 0.1N HCl at room temperature and it was diluted up to the mark with methanol. Then the sample of 20 µg/mL was prepared by diluting with mobile phase and it was analyzed.

The same study was repeated by preparing another sample and heating it for 15 mins. Sample was neutralized with 0.1N HCl at room temperature and it was diluted up to the mark with methanol. Then the sample of 20µg/mL was prepared from that and was analyzed.

Looking in the susceptibility of REM for base degradation, the experiment was performed without heating. Appropriate aliquots of stock solutions 2 mL of (1000 µg/mL) of REM was taken in 10 mL volumetric flasks and 2.5 mL of 0.1N NaOH was allowed to stand for 5mins was further neutralized with 0.1N HCl at room temperature and it was diluted up to the mark with methanol. The sample of 20µg/mL was prepared from that and was analyzed.

2.5.2. Acid Hydrolysis

To perform acid degradation study, appropriate aliquots of stock solutions 2mL of (1000 µg/mL) of REM was taken in 10 mL volumetric flasks and 2.5 mL of 0.1N HCl was added. Then the mixture was heated in a water bath at 70^{o}C for 1hr and allowed to cool to room temperature. The solution was neutralized with 0.1N NaOH and it was diluted up to the mark with methanol. Then the sample of 20µg/mL was prepared by diluting with mobile phase and it was analyzed.

The same study was repeated by preparing another sample and heating it for 30 mins at 70°C and allowed to cool to room temperature. The solution was then neutralized with 0.1N NaOH and it was diluted up to the mark with methanol. Then the sample of 20µg/mL was prepared from that and was analyzed.

2.5.3. Oxidative Stress Degradation

To perform oxidative stress degradation, appropriate aliquots of stock solutions 2 mL of (1000 µg/mL) REM was taken 10 mL volumetric flasks and 2.5 mL of 3% hydrogen peroxide was added. Then the mixture was heated in a water bath at 70ºC for 2 h and allowed to cool to room temperature and diluted up to the mark with methanol. Then the sample of 20µg/mL was prepared by diluting with mobile phase and it was analyzed.

2.5.4. Dry Heat Degradation

Analytically pure samples of REM was exposed in oven at 70ºC for 2h. The solids were allowed to cool and 10 mg of REM was weighed, transferred to volumetric flasks (10 mL) and dissolved in few mL of methanol. Volumes were made up to the mark with the methanol. Aliquots from the stock solutions of REM was appropriately diluted with mobile phase to obtain 20 µg/mL of REM. The chromatogram was recorded and sample was analyzed.

2.5.5. Photo Degradation

Analytically pure samples of REM was exposed to Sun light for 24 h. A 10 mg of REM was weighed, transferred to volumetric flasks (10 mL) and dissolved in few mL of methanol. Volumes were made up to the mark with the methanol. Aliquots from the stock solutions of REM was appropriately diluted with mobile phase to obtain 20 µg/mL of REM. The chromatograms were recorded using proposed method.

2.6. Analysis of Tablet Dosage Form

Twenty tablets were weighed and powdered. Powder equivalent to 25 mg REM was taken in 25 mL volumetric flask. Methanol was added to the above flask, and the flask was sonicated for 15 min. The solution was filtered using Whatman filter paper No. 45, and the volume was made up to the mark with methanol. Appropriate volume of the aliquot (1 mL) was transferred to a 10 mL volumetric flask, and the volume was made up to the mark with the methanol to obtain 100 µg/mL of REM. The aliquot was taken from above solution in 10 mL volumetric flask and diluted with mobile phase to obtain final concentration of 10 µg/mL REM. The solutions were sonicated and was injected in a system equilibrated with above chromatographic conditions. Peak area and retention time were obtained and quantification was carried out using regression equation.

3. Results and Discussion

3.1. Optimization of Mobile Phase

The objective of the method development was to obtain sharp peaks for active drug ingredient with less asymmetric factor and have resolution of more than 2 between drug and degradant peak. Different mobile phases (in combination of methanol & water) were tried to obtain sharp and well resolved peak of the drug. When the study was performed using mobile phase methanol:water (90:10 %, v/v), the drug (REM) was eluted before void peak at 2.1 min which was unacceptable. So, study was carried out using mobile phase methanol:water (80:20%, v/v), where the drug gave sharp and acceptable peak. When forced degradation was performed using same mobile phase REM and degradant peak were having resolution less than 1 which was not acceptable. So, study was performed using methanol : water (70:30%v/v) which gave two symmetric and well-resolved peaks for REM and its degradants and retention time for REM was found to be 10.4 min (Figure S2 and S3, see supporting information). So, methanol:water (70:30%, v/v) was selected as optimized mobile phase.

3.2. Method Validation

The calibration curves were obtained by plotting the peak area versus concentration over the range of 1– 25µg/mL with a correlation coefficient of 0.997. Linear regression equation was found to be y = 30.657x + 9.5316. Instrumental precision was determined by performing injection repeatability test. The RSD % values were found to be less than 1 which indicate that the method is repeatable. For the intra-day precision study, the RSD % values were found to be 0.23-1.3 and for the inter-day precision study the RSD % values were found to be 0.54-1.51. The low RSD % values indicate that the method is precise. Summary of validation parameters are shown in table 1.

Table 1. Summary of Validation Parameters

Parameters	**Results**
Linearity (µg/mL)	1-25
Detection limit (µg/mL)	0.21
Quantitation limit (µg/mL)	0.66
Accuracy (%)	98.29 % - 99.18 %
Intermediate Precision (%RSD)	
Intra-day (n = 3)	0.23-1.3
Inter-day (n = 3)	0.54 - 1.51
Instrument precision (%RSD)	
Repeatability (n=6)	0.84%
Specificity	Specific

The accuracy of the method was determined by calculating recoveries of REM by method of standard addition. The recoveries were found to be 98.29-99.18 % which indicated that the method is accurate. The detection limit for REM was found to be 0.2µg/mL, while quantitation limit was found to be 0.6µg/mL. The above data shows that a nanogram quantity of both the drugs can be accurately and precisely determined. Specificity studies were performed by preparing synthetic mixture using exicipients. No interference of excipients were observed which indicate that the method is specific. The low values of RSD obtained after introducing small, deliberate changes in parameters of the developed RP – LC method confirmed its robustness. The developed method was applied for the estimation of drug in tablet dosage form. The % amount of drug found was to be 98.2 ± 1.04.

Literature review revealed that HPTLC method [8] has been reported for the estimation of REM in bulk and tablet dosage form. The statistical comparison of assay results was carried out by F test. The F calculated value at 95% confidence interval were found to less than F tabulated value which indicates that there is no significant difference between two data set. Even the proposed HPLC method is stability indicating and has more sensitivity compared to reported HPTLC method.

3.3. Forced Degradation Study

Chromatogram of a base hydrolysis (0.1N NaOH) performed at 70ºC for 30mins showed complete degradation of drug with degradation peak at R_t 0.63 (Figure 1A).The study was repeated by heat exposure for 15 mins. It also showed complete degradation of REM with degradation product peak at R_t 6.6. (Figure 1B). Then the same study was performed without heating. The sample was allowed to stand for 5 mins and was neutralized and the peak of degradant and drug was observed to be well resolved at R_t 6.4 with resolution value of 7.72. (Figure 1C).

Chromatogram of acid hydrolysis (0.1 N HCl) performed at 70ºC for 1 hr degraded samples showed complete degradation of drug having degradation product peak at R_t 6.2 hence it was further repeated by heating the sample for 30 mins to obtain the resolved peak of drug and degradant. The degradation product peaks was found to be resolved at R_t 6.4 with resolution of 6.24 (Figure 2A). Oxidative stress degradation study performed using hydrogen peroxide and heating the sample for 2hrs at 70ºC showed that REM was found to be stable and least degradation was observed in this condition. The peak of degradant was observed at R_t 6.3 (Figure 2B).

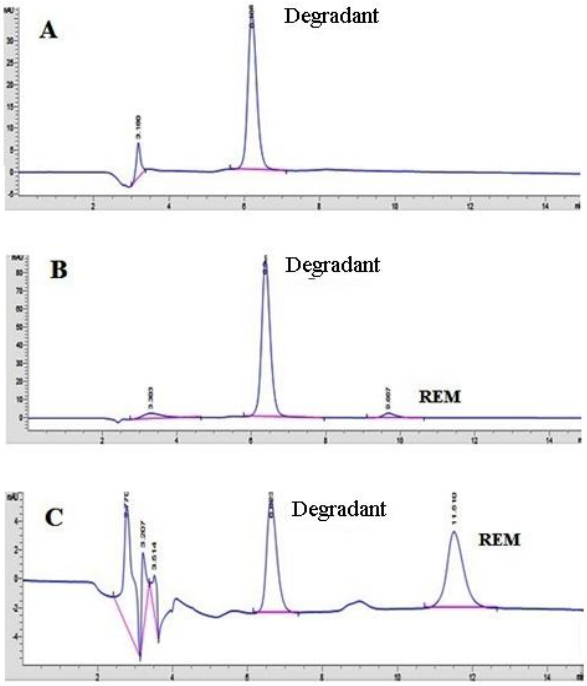

Figure 1. Chromatogram of **(A)** degradation studies in presence of base (0.1N NaOH, 30mins, 70ºC) **(B)** degradation studies in presence of base.(0.1N NaOH, 15mins., 70ºC) **(C)** degradation studies in presence of base (0.1N NaOH, 5 mins at Room temperature) using mobile phase Methanol : Water (70:30%v/v).

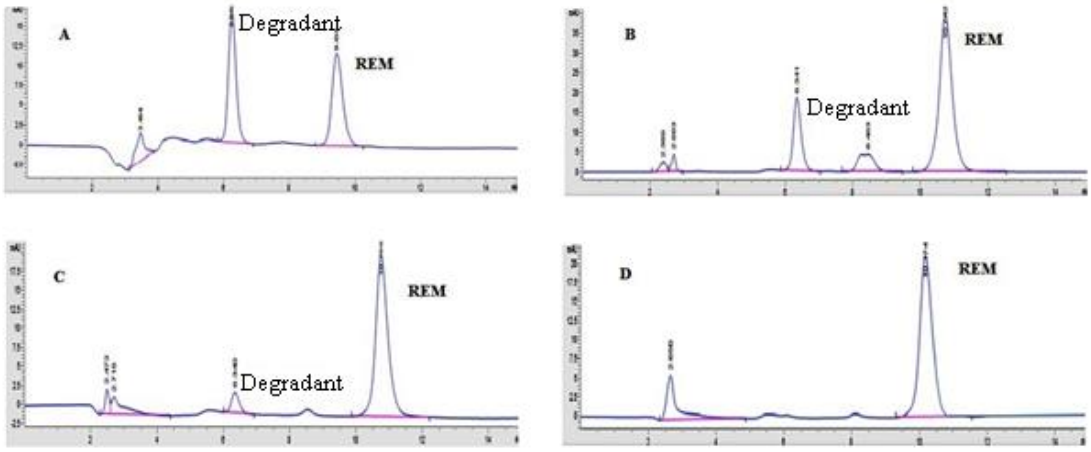

Figure 2. Chromatogram of **(A)** degradation studies in presence of acid (0.1N HCl, 30 mins., 70ºC) using mobile phase Methanol : Water (70:30%v/v) **(B)** degradation studies in presence of H_2O_2 (Heating 2Hr., 70ºC) using mobile phase Methanol : Water (70:30%v/v) **(C)** Dry Heat Degradation study **(D)** Photostability studies

REM was found to be quite stable to dry heat degradation conditions and samples showed degradation products peak at R_t 6.3 (Figure 2C). No degradation was observed under photolytic degradation condition hence drug was found to be stable under photolytic stress conditions (Figure 2D).The data of stability is further summarized in Table 2.

The degradation study indicated that REM was highly susceptible to acid and base hydrolysis and oxidative stress degradation. The drug was stable to dry heat and photolytic degradation conditions used

in the method. No degradation products from different stress conditions affected determination of REM. Peak purity was found to be more than 0.999 and graph is shown in Figure 3.

Table 2. Summary of forced degradation studies

Condition		Time	% Amt. of Drug	Rt of degradation products
Base	0.1 N NaOH	30 mins	-	6.31
	(70-80°C)	15 mins	-	6.60
	0.1 N NaOH	5 mins	25.64	6.62
	(RT#)			
Acid	0.1 N HCl	1hr	-	6.21
	(70-80°C)	30mins	42.45	6.24
Oxidative degradation	3% H_2O_2 (70-80°C)	2hr	89.76	6.34, 8.46
Dry heat	(70-80°C)	2hr	94.31	6.34
Photolytic	(Sunlight)	24hr	98.08	-

#RT: Room temparature

Remogliflozin etabonate undergo extensive ester hydrolysis in presence of acid - base and gets converted into active drug Remogliflozin. Literature study also revealed that REM is a pro-drug of remogliflozin [6], a benzylpyrazole glucoside-based inhibitor of renal sodium-glucose co-transporter subtype 2 (SGLT2) which possesses antihyperglycemic activity. This pro-drug upon the administration and absorption is converted to its active form remogliflozin which acts particularly on the sodium-glucose co-transporter subtype 2 (SGLT2).

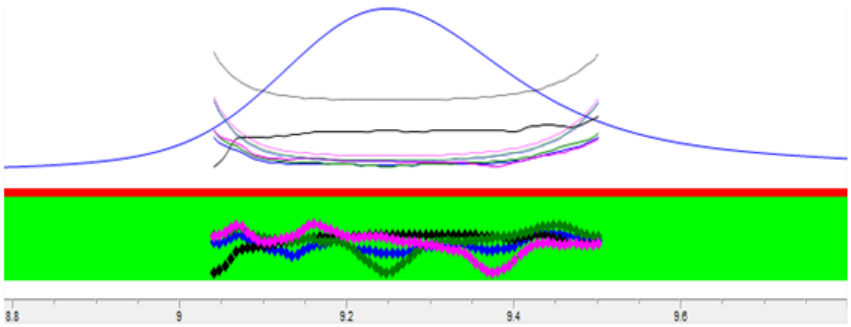

Figure 3. Peak purity data of stability studies of REM.

4. Conclusion

Reverse phase liguid chromatography (RP-LC) method has been developed for the estimation of REM in bulk and tablet dosage form. The method was validated and found to be accurate, precise, specific and sensitive. Low RSD % value obtained for validation parameter indicates the suitability of this method for routine analysis and quantitative determination of REM. Stability studies were performed and it was found that drug was highly susceptible to acid - base hydrolysis and gets converted into active drug metabolite remogliflozin. Degradants were well resolved from drug peak and no interference from degradants were observed which indicates the suitability of method for stability sample analysis. Compared to reported HPTLC method, the developed method is stability indicating and has more sensitivity. The method can be used for the routine analysis of bulk drug, formulation and stability samples.

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Supporting Information

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