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QBD STRESSED DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING HPTLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF REMOGLIFLOZIN ETABONATE AND TENELIGLIPTIN HYDROBROMIDE HYDRATE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A simple, selective, precise, and accurate chromatographic methods, namely High-performance thin-layer chromatography (HPTLC), were developed and validated for the simultaneous determination of Remogliflozin Etabonate and Teneligliptin Hydrobromide Hydrate. The developed HPTLC method was used for the separation and quantitation of the studied drugs on silica gel 60F254 TLC plates (20 cm × 10 cm) and a mobile phase consisting of methanol, ethyl acetate, and chloroform (7:2:1 v/v), chromatographic separation was accomplished. The CAMAG Linomat 5 applicator was used for the spotting, and a microliter syringe was used. The detection wavelength was 226 nm. The design space was created and process parameters such as the solvent front, chamber saturation period, and mobile phase proportion were optimized using the quality by design methodology. Well-resolved symmetric peaks were obtained using this designed chromatographic technique. Remogliflozin Etabonate and Teneligliptin Hydrobromide Hydrate were eluted at Rf 0.65 and 0.25, respectively. The ICH Q2 (R1) guideline was followed in the validation of this procedure. With r^2=0.9996 and 0.9999, respectively, the approach demonstrated linearity in the range of 2-10 µg per spot for Remogliflozin Etabonate and 0.2-1 µg per spot for Teneligliptin Hydrobromide Hydrate. The label claim pertaining to the sample recoveries indicated that there was no influence from formulation ingredients throughout the estimate process. The stressed materials were analyzed using the described approach, and forced degradation investigations were conducted.

Keywords: Remogliflozin Etabonate, Teneligliptin, Stability, Method, HPTLC

INTRODUCTION

Quality by design (QbD) approach has been educated by the Food and Drug Administration for the pharmaceutical development to ensure predefined quality attributes of the product. All together, the implementation of the QbD concept to the analytical method development leads to a more robust and repeatable method. QbD is described as "a systematic approach to development that begins with predefined objectives and emphasises product and process control, based on sound science and quality risk management" in the ICH Q8 (R2) standards. As a result, the variables in this study are discovered, their interactions are investigated, and eventually a technique with the best values for the variables is produced **[1]**. Stability studies are an essential part of the drug development program. In the most recent study, a central composite design (CCD) was employed to improve the HPTLC method's chromatographic conditions. Due to its adaptability, CCD was chosen and used to improve the chromatographic conditions by better understanding the individual and combined effects of variables. The goal of this study was to create a straightforward, speedy, accurate, and precise HPTLC method for the quantitative analysis of RGE and TG utilizing a DOE methodology, and to validate the method in accordance with ICH requirements. The molecular name for Remogliflozin Etabonate (RGE) is 5-Methyl-4-[4-(1-methylethoxy) benzyl]-1-(1-methylethyl)-1H-pyrazol-3-yl 6-O-(ethoxycarbonyl)-β-D-glucopyranoside and it works by removing excess sugar from body through urine. It is soluble in methanol and not official in any Pharmacopoeia. The molecular name for Teneligliptin Hydrobromide Hydrate (TG) is [(2*S*, 4*S*)-4-[4-(5-methyl-2-phenylpyrazol-3-yl) piperazin-1-yl] pyrrolidin-2-yl]-(1, 3-thiazolidin-3yl)methanone; hydrate; pentahydrobromide and it works by increasing the release of insulin from the pancreas and decreasing the hormones that raise blood sugar levels. This reduces the fasting and post-meal sugar levels. It is also soluble in methanol and official in Indian Pharmacopoeia **[2-5]**.

Literature survey revealed that several analytical methods have been reported for RGE and TG individually and in combination with other drugs like ultraviolet spectrophotometry **[3-11]**, high-performance liquid chromatography (HPLC) **[12-20]**, HPTLC **[21]**, UPLC **[22, 23]**, and RP-UFLC **[24]**, but no high performance thin layer chromatography method is reported for simultaneous estimation of both RGE and TG in combination. The spectrophotometric and high performance liquid chromatographic method is available for simultaneous

estimation of both the drugs but in both the method, the concentration used is high and also high detection and quantification limit **[25]**. Thus, a need for a method arises, for estimating the drugs in combination that is simple, rapid, accurate, repeatable, robust and required less chemicals for routine analysis. The ICH Q1A (R2) guideline entitled "Stability testing of new drug substances and products" requires stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. An ideal stability-indicating method is the one that resolves the drug and its degradation products efficiently. So the aim of the present work was to develop and validate a stability-indicating HPTLC method for simultaneous estimation of RGE and TG in a pharmaceutical dosage form within the QbD framework.

Materials and Methods

The RGE reference standard was procured from Glenmark Pharmaceuticals Ltd, Ranjool, Sikkim and TG reference standard was procured from Alidhara Pharmaceutical Pvt Ltd, Vadodara, Gujarat. The commercial formulation Zita plus R tablets containing 100 mg of RGE and 10 mg of TG was procured from a local pharmacy. HPLC grade methanol, chloroform, ethyl acetate and water were procured from Merck, India.

The HPTLC system by CAMAG equipped with a win CATS software was used for chromatographic separation. Separation was carried out on a pre coated aluminum plates with silica gel 60 F-254 (20 cm × 10 cm). CAMAG linomat 5 applicator with microliter syringe was used for application. The Rate of application was 200 nL/s. The mobile phase consisting Chloroform: Ethyl Acetate: Methanol (7:2:1 v/v) was used for separation. Detection wavelength selected for the estimation of the two drugs was 226 nm.

A three-factorial, rotatable central composite statistical experimental design was employed using 17 experimental runs that included 1 center points. The independent variables, such as the Ethyl acetate content in the mobile phase (A), distance travelled (B) and chamber saturation time (C), and the responses for all 17 optimized trial experimental runs are summarized in **Table 1**. The mobile phase is composed of three components but amongst them ethyl acetate content is selected as variable because minor change in ethyl acetate content gives major effect on Rf. During model selection, the best-fitted models for the Rf value of RGE and TG were a linear and quadratic model, respectively, based on the lowest PRESS value and adjusted R2 value closer to 1.

Table 1: Central composite design arrangement and responses.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A:Ethyl acetate (ml)	B:solvent front (cm)	C:Chamber saturation (min)	Rf of TG	Rf of RGE
1	2	90	20	0.05	0.55
2	3	70	25	0.17	0.67
3	4	90	20	0.36	0.86
4	3	70	15	0.53	0.89
5	3	90	25	0.09	0.59
6	3	80	20	0.15	0.65
7	2	70	20	0.12	0.62
8	3	80	20	0.15	0.65
9	4	80	25	0.94	0.96
10	3	80	20	0.15	0.65
11	4	70	20	0.97	0.97
12	2	80	25	0.07	0.57
13	3	80	20	0.15	0.65
14	4	80	15	0.75	0.9
15	2	80	15	0.26	0.76
16	3	80	20	0.15	0.65
17	3	90	15	0.13	0.63

Preparation of standard solution

Ten milligrams of standard RGE and TG were accurately weighed, transferred to two separate 10 ml volumetric flasks, dissolved in methanol and brought to volume with methanol to obtain a solution containing 1000 µg/ml of solution. Aliquots of the stock solutions were appropriately diluted with methanol to obtain working standards of 6 µg per spot RGE and 0.6 µg per spot TG.

Chromatographic Procedure

Standard solutions of different concentrations were spotted with a micro-syringe in the form of bands having a band width of 6 mm on a pre-coated silica gel aluminium Plate 60 F254 using a Camag Linomat 5 sample applicator. Linear ascending development was carried out in a twin trough glass chamber. The mobile phase consisted of Chloroform: Ethyl Acetate: Methanol (7:2:1 v/v). The optimized chamber saturation time before chromatographic development was 30 min at room temperature. The length of the chromatographic run was 7 cm. Subsequent to the development, HPTLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed using a Camag TLC scanner 4 with the win CATS software. All measurements were made in the reflectance–absorbance mode at 226 nm, with a slit dimension of 6.00 mm × 0.30 mm (micro), scanning speed of 20 mm/s. The source of radiation was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. The concentrations of both drugs were determined based on the intensities of diffusely reflected lights, and the data were evaluated using an ordinary linear regression analysis of peak areas.

Software aided method optimization

A central composite design (CCD) was used to optimize the compositional parameters and evaluate main effect, interaction effects and quadratic effects of the factors on the retardation factor (Rf) of both drugs. CCD is a useful aspect of the response surface methodology to explore quadratic response surfaces and construct polynomial models without the need for a complete three-level factorial experiment. The selection of critical factors and ranges examined for optimization was based on preliminary univariate studies of method development and chromatographic intuition. The composition of the mobile phase refers to the volume of ethyl acetate with respect to the total volume of the mobile phase. Seventeen experiments with one center points were conducted by selecting three factors; the ethyl acetate content in the mobile phase (A), the solvent front (B) and chamber saturation time (C) and the Rf of TG and RGE were the responses selected for both drugs depicted in **Table 1**.

The model was also validated with an analysis of variance (ANOVA) using the Design Expert software and the results are presented in **Table 2**. Significant effects had a P value less than 0.05. An adequate precision, a measure of the signal (response) to noise ratio, greater than 4 is desirable, and the obtained ratio for both drugs indicated an adequate signal. The **Model F-value** of 48.74 and 33.06 implies the model is significant.

Table 2: Predicted response models and statistical parameters obtained from the ANOVA for CCD

Response	Type of Model	Adjusted R^2	Predicterd R^2	Model P value	% CV	Adequate Precision
Rf of TG	Quadratic	0.9475	0.6339	0.0004	4.48	16.9346
Rf of RGE	Quadratic	0.9641	0.7828	0.0008	18.80	20.2736

The adjusted R-square values were high, indicating a good relationship between the experimental data and those of the fitted models. Here, the adjusted R2 values were well within the acceptable limit which indicated that the experimental data fitted polynomial equations well. VIFs less than 10 are tolerable **(Table 3 and 4)**.

Table 3: Coefficients in Terms of Coded Factors for Response 1

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.1573	1	0.0272	0.0931	0.2215	
A-Ethyl acetate	0.2387	1	0.0224	0.1858	0.2917	1.22
B-Solvent front	-0.1646	1	0.0230	-0.2191	-0.1102	1.29
C-Chamber Saturation Time	-0.1271	1	0.0262	-0.1890	-0.0653	1.44
AB	-0.1000	1	0.0287	-0.1679	-0.0321	1.00
AC	0.2052	1	0.0382	0.1149	0.2955	1.30
BC	0.4134	1	0.0818	0.2199	0.6069	5.02
A^2	0.1721	1	0.0395	0.0788	0.2655	2.00
B^2	0.0959	1	0.0443	-0.0088	0.2005	2.52
C^2	0.2777	1	0.0467	0.1673	0.3881	2.72

Table 4. Coefficients in Terms of Coded Factors for Response 2

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	**0.6556**	**1**	**0.0152**	**0.6196**	**0.6916**	
A-Ethyl acetate	**0.1102**	**1**	**0.0126**	**0.0805**	**0.1400**	**1.22**
B-Solvent front	**-0.0792**	**1**	**0.0129**	**-0.1097**	**-0.0486**	**1.29**
C-Chamber Saturation Time	**-0.0768**	**1**	**0.0147**	**-0.1115**	**-0.0421**	**1.44**
AB	**0.0250**	**1**	**0.0161**	**-0.0131**	**0.0631**	**1.00**
AC	**0.0989**	**1**	**0.0214**	**0.0483**	**0.1496**	**1.30**
BC	**0.2352**	**1**	**0.0459**	**0.1266**	**0.3437**	**5.02**
A^2	**0.0523**	**1**	**0.0222**	**-0.0001**	**0.1047**	**2.00**
B^2	**0.0764**	**1**	**0.0248**	**0.0177**	**0.1351**	**2.52**
C^2	**0.1324**	**1**	**0.0262**	**0.0704**	**0.1943**	**2.72**

Final Equation in Terms of Coded Factors

- R1 = +0.1573 + 0.2387A - 0.1646B - 0.1271C – 0.10000AB + 0.2052 AC + 0.4134 BC + 0.1721 A^2 + $0.0959B^2$ + $0.2777C^2$
- R2 = +0.6556 + 0.1102A – 0.0792B – 0.0768C – 0.0250AB + 0.0989AC + 0.2352BC +$0.0523A^2$ + $0.0764B^2$ + $0.1324C^2$

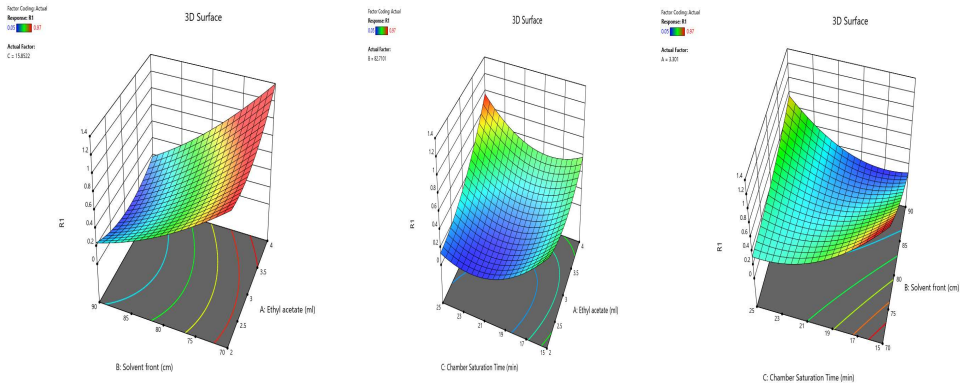

Figure 1: Three-dimensional plots of the Response 1 (a) variation in the Rf of Teneligliptin Hydrobromide Hydrate (TG) as a function of A and B for a fixed value of C; (b) variation in the Rf of Teneligliptin Hydrobromide Hydrate (TG) as a function of A and C for a fixed value of B; (c) variation in the Rf of Teneligliptin Hydrobromide Hydrate (TG) as a function of B and C for a fixed value of A

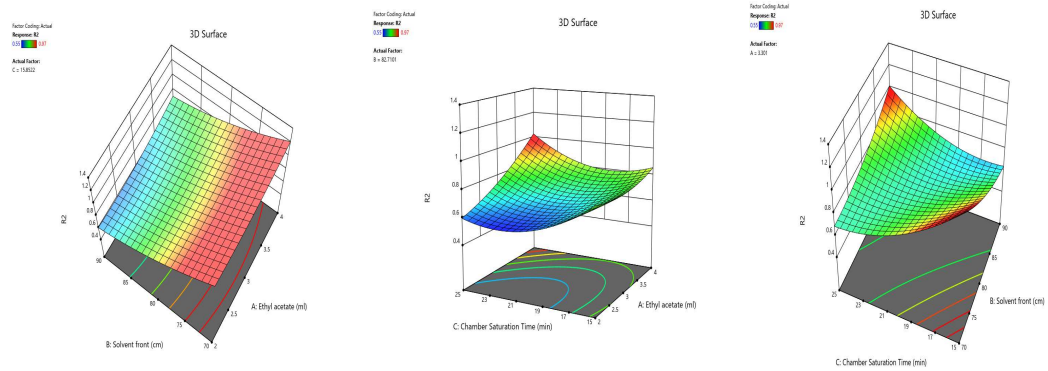

Figure 2: Three-dimensional plots of the RSM for Response 2 (a) variation in the Rf of Remogliflozin Etabonate (RGE) as a function of A and B for a fixed value of C; (b) variation in the Rf of Remogliflozin Etabonate (RGE) as a function of A and C for a fixed value of B; (c) variation in the Rf of Remogliflozin Etabonate (RGE) as a function of B and C for a fixed value of A

The optimum conditions of separation were estimated using Derringer's desirability function. During the numerical optimization, the targets of individual factors and responses were fixed. Of the 17 different solutions of the optimization provided by the software, two conditions that have a desirability near 1 were selected. The response surface obtained for the maximum Derringer's desirability function is presented in **Figure 1 and Figure 2**. The selected optimized composition for the final HPTLC analysis was Chloroform: Ethyl Acetate: Methanol (7:2:1 v/v). Under the optimized conditions, the HPTLC densitogram showed an Rf of 0.65 for RGE (6 µg per spot) and 0.25 for TG (0.6 µg per spot) and is depicted in **Figure 3**.

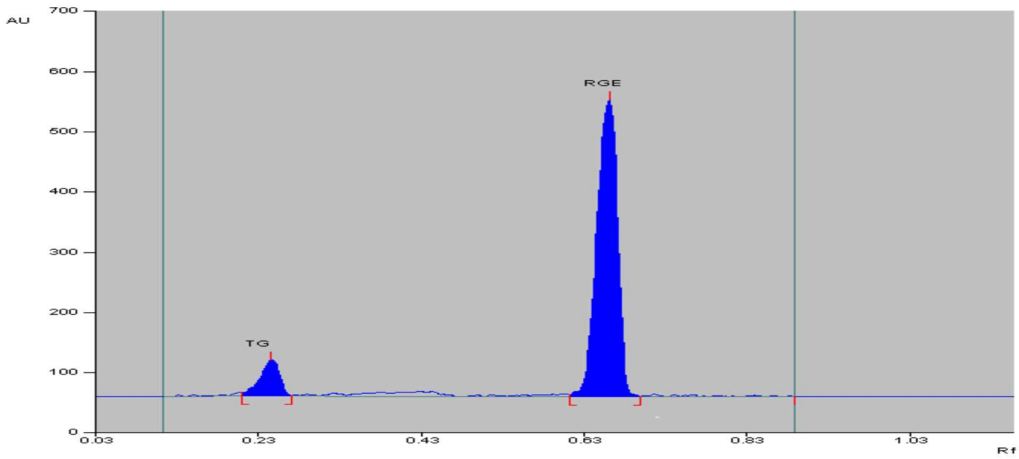

Figure 3: HPTLC densitogram under optimized conditions showing Rf of TG 0.25 and Rf of RGE 0.65

RESULT AND DISCUSSION

Method validation [18-23]

The accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), specificity, and robustness of HPTLC method were validated in accordance with the ICH Q2 (R1) guideline.

Linearity and Range

The linearity was observed by analyzing calibration curve in the range of 2–10 µg per spot and 0.2–1 µg per spot for RGE and TG respectively (n = 6). The calibration curve of Area vs. respective concentration was plotted and correlation coefficient and regression line equations for RGE and TG were mention in **Figure 4**.

Precision

The experiment was repeated six times by using same concentration from homogeneous mixture for repeatability, three times in one day (intra-day precision), and the average % RSD values of the results were calculated. Similarly, the experiment was repeated on three different days (inter-day precision), and the average % RSD values for the peak areas of RGE and TG were calculated. The intra-day and inter-day precision is expressed in terms of % RSD

and was less than 2, confirming the precision of the method **(Table 5)**.

Recovery studies

The accuracy was calculated for both drugs by spotting 4000 ng/band of RGE and 400 ng/band of TG in 100 ml volumetric flasks. In other three 100 ml volumetric flasks add 80%, 100% and 120% of RGE and TG respectively. Each solution was scanned between 200 nm to 400 nm. The amount of RGE, TG and % recoveries was calculated **(Table 5)**.

Specificity

The chromatogram of the pharmaceutical formulation obtained using the developed method showed only two peaks at Rf of 0.65 and 0.25 for RGE and TG, respectively, and was found to be at the same Rf for both standard drugs. The peak purity of both drugs in pharmaceutical dosage form was confirmed by comparing the overlaid spectra at the peak start, peak apex and peak end positions of the band. The results shown in Table 4 demonstrate that the purity exceeded 0.999 for all peaks, indicating the specificity of method in the presence of various excipients **(Figure 6)**.

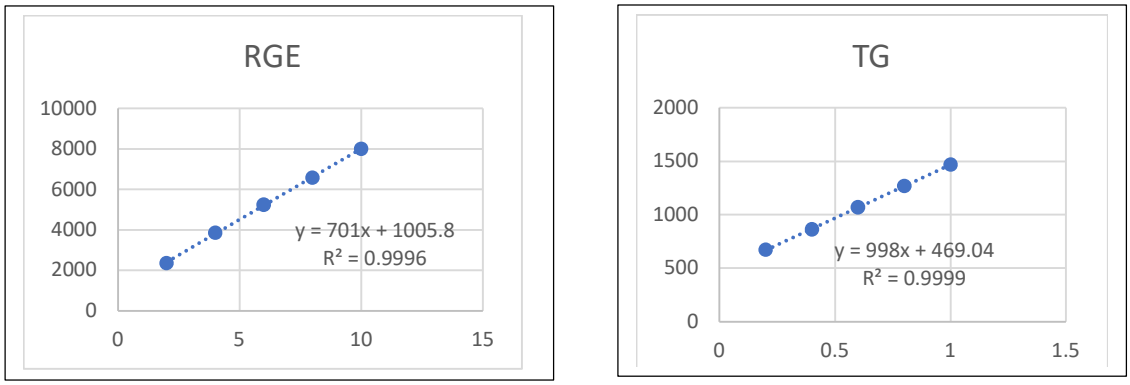

Figure 4: Calibration curve of Remogliflozin Etabonate and Teneligliptin Hydrobromide Hydrate

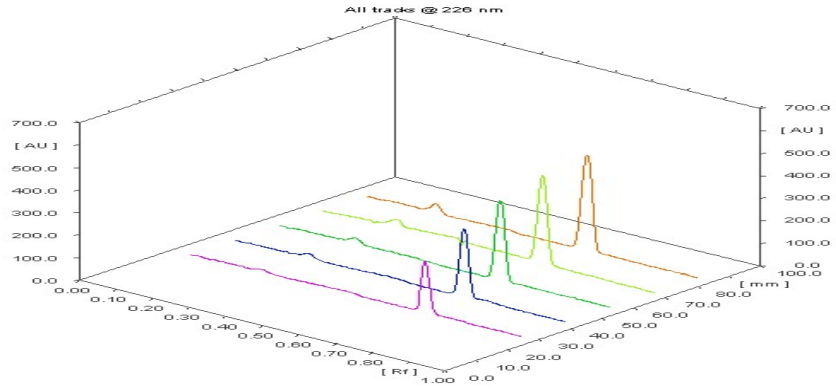

Figure 5: Linearity of RGE and TG by 3D Densitogram

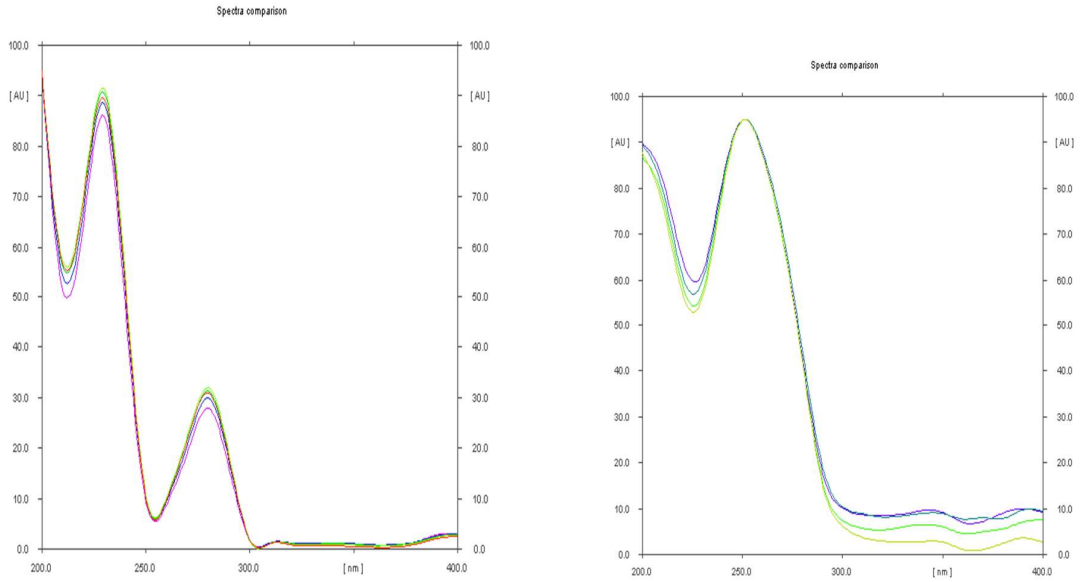

Figure 6: Overlain peak purity spectra of (a) RGE and (b) TG with the corresponding standard

Robustness

A deliberate change in various parameters, such as change in chamber saturation time and wavelength, produced %Relative standard deviations of the peak area of less than 2%, indicating the robustness of the method **(Table 5)**.

LOD and LOQ

The LOD and LOQ of the developed method were found to be 0.04087 and 0.1238 µg per spot respectively, for RGE, and 0.0287 and 0.0870 µg per spot, respectively, for TG, indicating the sensitivity of the proposed method **(Table 5)**.

Analysis of marketed dosage form

The analysis of the tablet formulation containing 100 mg RGE and 10 mg TG showed good recovery. Specifically, the percentages were 100.76% for RGE and 99.81% for TG, indicating that the method can be used for routine quality control when testing the tablet dosage formulation. The %RSD value was found to be less than 2 **(Table 5)**.

Table 5: Analytical validation parameters for RGE and TG using the HPTLC method

Parameters		HPTLC method	
		RGE	TG
Concentration (µg per spot)		2-10	0.2-1
Slope		0.701	0.998
Intercept		1005.8	469.04
Correlation coefficient(R^2)		0.9996	0.9999
LOD (µg per spot)		0.0385	0.0325
LOQ (µg per spot)		0.1167	0.0986
Precision(%RSD)	Repeatability	0.23	0.93
	Intraday	0.14-0.36	0.43-1.01
	Interday	0.11-0.34	0.43-0.97
Accuracy (% recovery)		98.97 ± 100.07	100.06 ± 100.88
%Assay ± SD		100.76 ± 0.10	99.81 ± 0.74

Forced Degradation study

Forced degradation studies provide the approach to analyze the stability of drug samples. The ICH guidelines that are applicable to forced degradation studies are ICH Q1A (stability testing of new drug substances and products) and ICH Q1B (photostability testing of new drug substances and products) **[22]**.

Acid degradation

Acid degradation study was carried out by dissolving the drug in 0.1 N HCl solution to get the stock solution of 1000 µg/ml concentration and refluxing it at 60°C in for 1 hour to avoid any possible side effects of light. Solution was applied on a precoated plate of TLC after dilution to achieve concentration of 6 µg per spot for RGE and 0.6 µg per spot for TG and then chromatogram was run.

Alkaline degradation

Alkaline degradation study was carried out by dissolving the drug in 0.1 N NaOH solution to get the stock solution of 1000 µg/ml concentration and refluxing it at 60°C in for 1 hour to avoid any possible side effects of light. Solution was applied on a precoated plate of TLC after dilution to achieve concentration of 6 µg per spot for RGE and 0.6 µg per spot for TG and then chromatogram was run.

Oxidative degradation

Oxidative degradation study was carried out by dissolving the drug in 3% H_2O_2 solution to get the stock solution of 1000 µg/ml concentration and refluxing it at 60°C in for 1 hour to avoid any possible side effects of light. Solution was applied on a precoated plate of TLC after dilution to achieve concentration 6 µg per spot for RGE and 0.6 µg per spot for TG and then chromatogram was run.

Thermal degradation

For thermal degradation, drugs were kept in oven at 60°C for 4 hours, and it's of 1000 µg/ml solution was prepared in methanol. Solution was applied on a precoated plate of TLC after dilution to achieve concentration of 6 µg per spot for RGE and 0.6 µg per spot for TG and then chromatogram was run.

Photochemical degradation

For photochemical degradation study, the drugs was exposed in direct sunlight for 48 hours, after subsequent dilutions with methanol it was applied on plate to get concentration of 6 µg per spot for RGE and 0.6 µg per spot for TG and then chromatogram was run.

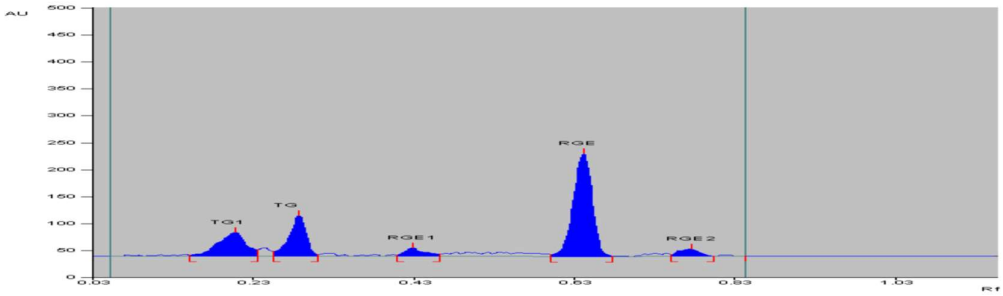

Figure 7 (a): Acid Degradation in 0.1 N HCl at 60°C and 1 hr for RGE and TG

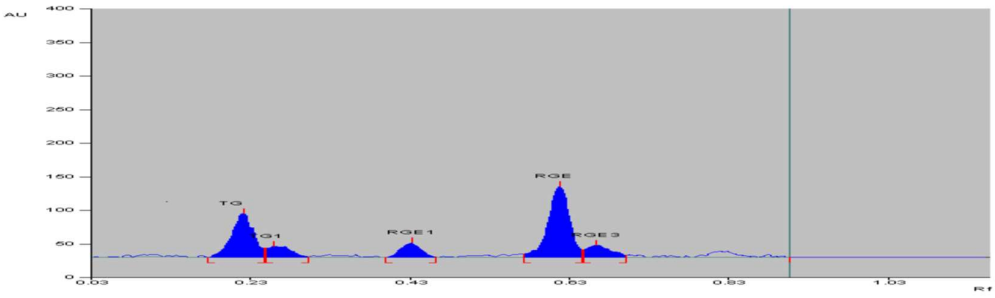

Figure 7 (b): Base Degradation in 0.1 N NaOH at 60°C and 1 hr for RGE and TG

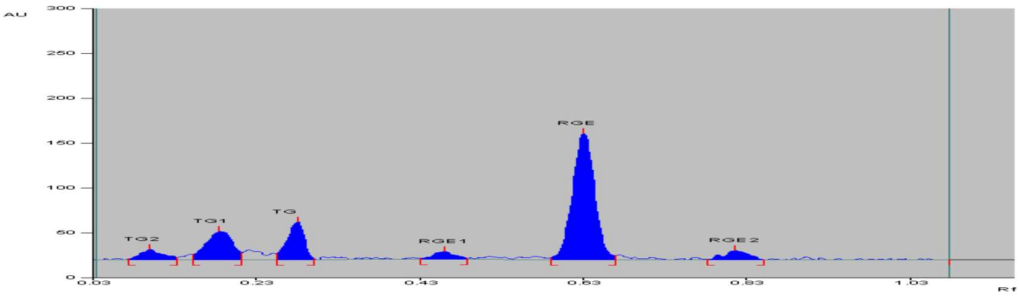

Figure 7 (c): Oxidative Degradation in 3% H_2O_2 at 60°C and 1 hr for RGE and TG

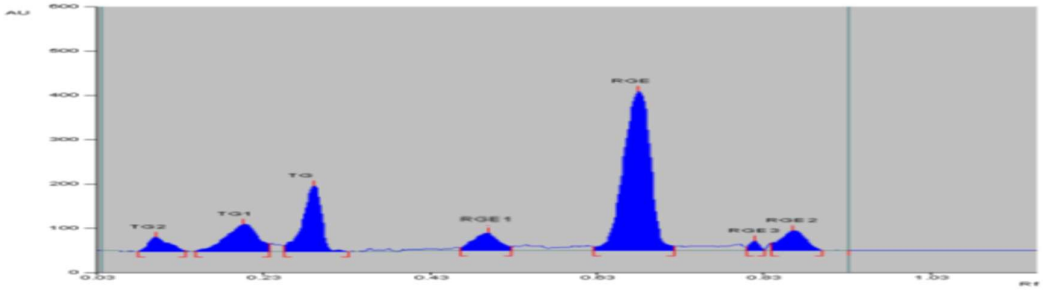

Figure 7 (d): Thermal Degradation in 60°C and 4 hr for RGE and TG

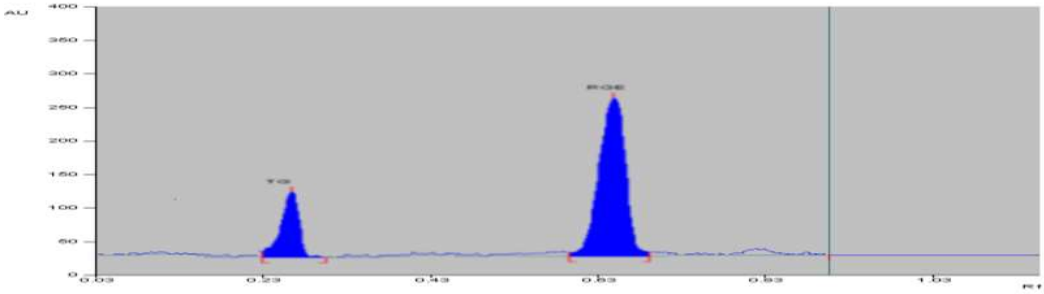

Figure 7 (e): Photolytic Degradation in sunlight for 48 hr for RGE and TG

Table 6: Stability studies for the developed method

Degradation condition	% degradation	
	RGE	TG
Acid Degradation	8.62 %	11.15 %
Base Degradation	15.65 %	10.71 %
Oxidation	7.99 %	17.57 %
Thermal	11.11 %	17.11 %
Photolytic	-	-

CONCLUSION

The current treatment for diabetes mellitus consists of remogliflozin etabonate and teneligliptin hydrobromide hydrochloride. A stability indicating high performance thin layer chromatography (HPTLC) method was developed and validated for the determination of Remogliflozin Etabonate and teneligliptin hydrobromide hydrochloride in formulations on pre-coated silica gel HPTLC plates. The mobile phase used in this method was chloroform: ethyl acetate: methanol (7:2:1 v/v/v), and densitometric detection was achieved at 226 nm. This was done because there are no reported methods for the simultaneous estimation. It was discovered that the created procedure was easy to use, quick, sensitive, selective, and appropriate for determining remogliflozin etabonate and teneligliptin hydrobromide hydrochloride at the same time. Higher mobile phase pH, larger sample capacity, shorter run times, minimal solution consumption, the ability to analyze the sample and standard simultaneously on the same plate, short system equilibrium times, multiple or repeated chromatogram scanning, and the absence of pre-treatments for solvents like filtration and degassing are just a few of the benefits that the HPTLC method has over liquid chromatographic methods. The drugs can be used for the simultaneous estimation of remogliflozin etabonate and teneligliptin hydrobromide hydrochloride and their degradation products in stability samples in the industry, according to the stability indicating properties established in accordance with the recommendations of the ICH guidelines.

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