COMPARISON OF ACID DEGRADANT PRODUCT WITH METABOLIC PATHWAY OF REMOGLIFLOZIN ETABONATE IN DEVELOPED AND VALIDATED RP-HPTLC METHOD

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ABSTRACT

Forced degradation studies in tablet and bulk formulations of remogliflozin etabonate have been developed using a precise and sensitive high performance thin layer chromatographic method. The silica gel RP-18 F_{254S} plates were used as the stationary phase and ACN: water: ammonia solution (8:2:0.5 V/V/V) was used as the mobile phase for estimation. The proposed method was successfully validated, showing the R_f of the drug as 0.72 at 229 nm. The method was observed to be linear in the range of 500-3000 ng band^{-1} and then degradation was estimated by forced degradation pathway. In the forced degradation studies, the drug was found to be highly susceptible to both acid and base, including oxidative conditions, providing an active metabolite when remaining in contact in stressed conditions for a short time (30 min), while in longer duration (36 h), the drug provided another metabolite which on characterization was found to be inactive and predicted to be reported in the metabolic pathway.

Keywords: Remogliflozin etabonate, HPTLC, Validation, Forced degradation studies

Abbreviations

HPTLC - High performance thin layer chromatography, R_f - Retardation factor, RSD - Relative standard deviation, LOD - Limit of detection, LOQ - Limit of quantification, UDP - Uridine diphosphate, ng - Nanogram

INTRODUCTION

The active form of remogliflozin etabonate (REM) (Fig. 1), a prodrug, is remogliflozin, which binds to subtype 2 of the sodium-glucose co-transporter (SGLT2) enzyme, which is basically used for the treatment of obese diabetes mellitus type-2[1]. The SGLT2 accelerates the elimination, via reabsorption, of sugar from the kidneys and additionally provides effects on controlling or lowering body weight and controlling the systolic pressure of the blood. The literature also reveals the use of REM in polycystic ovary syndrome[2].

The metabolic pathway of REM was designed to investigate the pharmacokinetic properties and influence of enzymes on REM and its metabolites to understand the risks of drug interactions[3]. As per pathway, the inactive form of REM was converted to the active form in the presence of esterase. The inactive product GSK1132678 was observed to be formed by the oxidative process of the hydroxyl group of glucose result to form glucuronic acid in the human liver cytosol in the presence of UDP-glucose dehydrogenase. Further, in the presence of glucuronosyltranferease (UGT), major metabolite (50%) was observed to form GSK1997711. The part of metabolism is depicted (Fig. 1) to compare the structure of final degradant products[4-5].

In Pharmacopoeias REM is not official, and quantitative analysis literature has primarily focused on the combination of REM with other drugs such as metformin. Analysis of the single form of REM in blood and plasma has been reported by Ultra Violet-visible (UV) spectroscopy, high performance liquid chromatography (HPLC), coupled with mass spectrometric methods, but the least method is available with high performance thin layer chromatography (HPTLC) in single form[6-9].

Several Pharmacopoeias also accepted the HPTLC monographs for estimation of the impurities of the drug, hence the current study also focused on the same. Compared to other liquid chromatography methods, the

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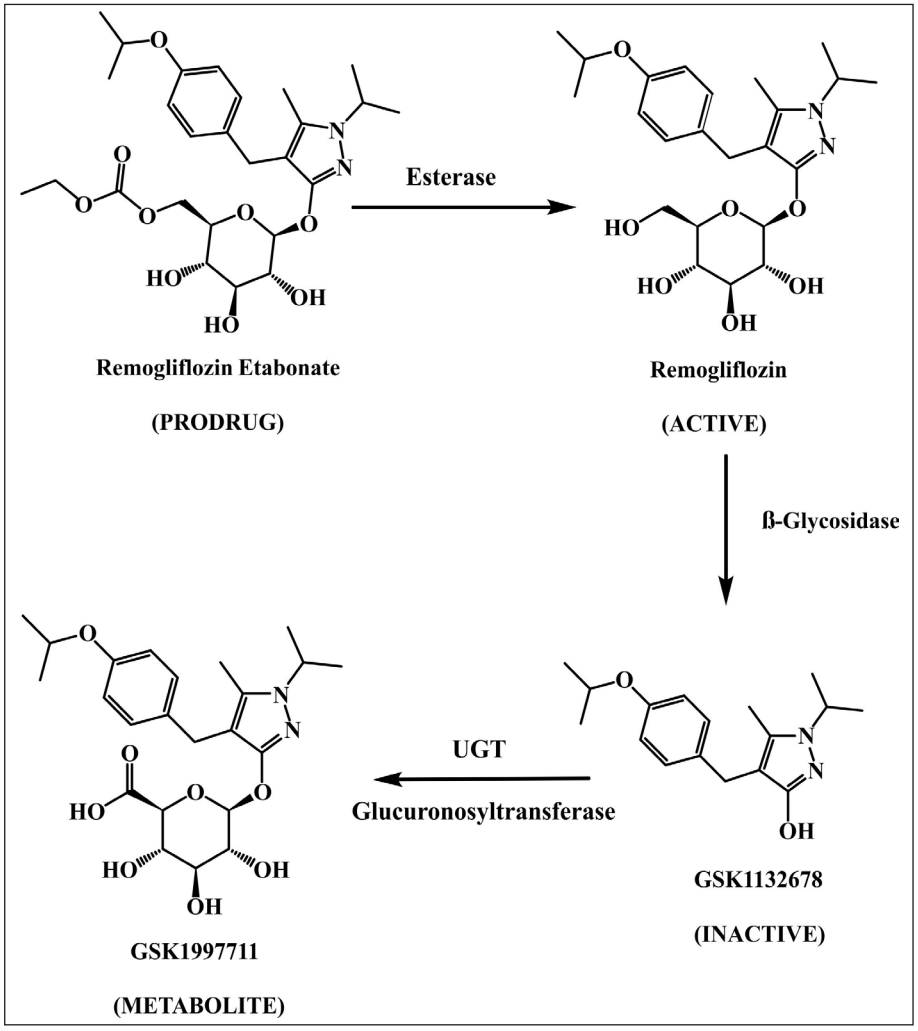


Fig. 1: Metabolic pathway of REM and formation of active drug and inactive metabolite[4]

HPTLC method is found to be more suitable with reduced analysis time, sample preparation and mobile phase[10-12]. The increased accuracy and precision with degradation studies in HPTLC is a result of densitometric scanning. So, in the present study, we discussed the development, validation, and stability indicators of the HPTLC method for estimation of REM.

MATERIALS AND METHODS

HPTLC instruments

CAMAG (Switzerland) with a Linomat V applicator, Hamilton syringe (100 µL), CAMAG Wincats programme (Version 1.4.10), CAMAG twin trough chamber (10×10 cm, 20×10 cm), and ultra-sonicator (ENERTECH Electronics Pvt. Ltd. (Mumbai, Maharashtra), were used in the study. The pre-coated aluminium HPTLC plates (10 × 10 cm) with silica gel RP-18 F_{254S} with a thickness of 250 µm were used as the stationary phase (E. Merck, Germany). For densitometric scanning, the CAMAG TLC Scanner III was used.

Chemicals and reagents

A REM sample (99.40% w/w pure, as per COA) was procured from Glenmark Pharmaceuticals Ltd., Nashik, Maharashtra, India. HPLC grade acetonitrile (ACN), methanol (HPLC grade), double distilled water, and ammonia solution (25%) were procured from Merck Chemicals Pvt. Ltd., Mumbai, India. Zucator (REM 100 mg) brand marketed by Torrent Pharmaceuticals Ltd., Mehsana, Gujarat, India, under license from Glenmark Pharmaceuticals Ltd., Sikkim, India was procured from local pharmacy.

Sample application

Initially, the plates were washed with methanol and dried at 110 °C. The samples of REM (Standard and formulation) were applied to pre coated silica gel F_{254S} plates in the form of 6 mM bands with a distance of 9 mM between two bands. The bands were applied using a continuous drying stream of nitrogen gas.

Optimization of mobile phase

Various solvent mixtures were tried for the development of an appropriate mobile phase. The acceptability of solvent mixtures were determined by the factors like lack of tailing, spot shape, sensitivity of the assay and time required for development. Different TLC chamber saturation times were also tried. The linear development was performed in the Automatic Developing Chamber (ADC2) with ACN: water: ammonia solution (8:2:0.5 V/V/V) as a mobile phase. The saturation time of chamber is 30 minutes at 25 ± 1°C and 35 ± 5% relative humidity control. The distance and time for the development was 80 mM and 11 min, respectively, in the ADC2 chamber.

Densitometric analysis

The scanning was performed using a densitometric TLC scanner III in absorbance-reflectance mode at 229 nm after scanning between 400-200 nm with deuterium

lamps. The slits were kept to a size of 6.0 × 0.45 mM, and the scanning speed was set to 20 mM per second. For each generated band, the area and height of the peak were determined, and a regression equation was created by graphing peak areas versus concentration.

Preparation of stock standard solution

REM (50 mg) was accurately weighed and was transferred into a volumetric flask of 10 mL with a few mL of methanol and sonicated for 10 min. Further, dilution with methanol up to the mark provided a concentration of 5000 µg mL^{-1}.

Calibration curve

In triplicate, varying volumes of standard stock solution (5-30 µL $band^{-1}$) were applied to a TLC plate, and calibration curves were generated by graphing peak areas versus concentration ranging from 500-3000 ng $band^{-1}$.

Validation

Guideline Q2 (R1) of the International Conference on Harmonization (ICH) for the validation of analytical methods was followed[13-14].

Linearity of calibration curves

Linearity of the peak area response was determined by making six measurements in the range of 500-3000 ng $band^{-1}$ for REM. Calibration curves were obtained by graphing the peak area verses different concentrations of the REM.

Accuracy

Accuracy was determined as percent recovery by the standard addition method. The pre-analyzed samples (100 ng $band^{-1}$) were spiked with 80%, 100% and 120% of the standard, and the mixtures were reanalyzed in triplicate using the proposed method. At each concentration level, the percent recovery and RSD were determined.

Intermediate precision

Inter-day precision observes to applying the analytical method in 3 days over a specific period of time by the same analyst with unchanged instrument estimating drug solutions of REM (1000, 1500, 2000 ng $band^{-1}$). Intra-day was determined by the application of the analytical method within a day at different periods of REM (1000, 1500, 2000 ng $band^{-1}$) of time by the same analyst with the same instrument. From the analysis of developed densitograms, peak area was obtained, and variability of data was calculated in terms of %RSD.

Repeatability

The repeatability of the method was assessed by applying a band of REM (1500 ng $band^{-1}$) six times on an HPTLC plate. The peak areas were estimated after the plates were developed. The same spot was scanned six times for repeatability study of the scanner, the peak area was calculated, and the variability in the results was examined.

Sensitivity

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value. As per ICH guidelines:

LOD = 3.3*(SD of the response / slope)

LOQ = 10*(SD of the response / slope)

Robustness

Robustness was studied in triplicate at 1500 ng $band^{-1}$ by making small changes to the volume of wavelength, composition of the mobile phase, developing distance and developed densitogram were analyzed.

Forced degradation study

Forced degradation experiments were carried out to govern the inherent stability of the drug molecule and potential breakdown pathways as per ICH guideline Q1A (R2) and Q1B using different stress conditions like acid and base hydrolysis, thermal degradation and oxidative hydrolysis conditions.

Alkali hydrolysis

The alkali (base) hydrolysis was performed by transferring 1 mL of stock solution (1000 µg mL^{-1}) of REM and 0.1 N NaOH solutions into a 10 mL volumetric flask. Before neutralisation and dilution with methanol, the solution was kept at room temperature for 30 min. The solution sample was analyzed and the densitogram of REM was observed to check degradation.

Acid hydrolysis

The acid hydrolysis was performed by transferring 1 mL stock solution (1000 µg mL^{-1}) of REM and 0.1 N HCl solutions in 10 mL volumetric flask. Before neutralization and dilution with methanol, the solution was heated to 70 ^{0}C for 30 min and was then kept at room temperature to get cooled. The solution sample was analyzed and the densitogram of REM was observed to check degradation.

Oxidative stress degradation

The oxidative degradation was performed by transferring 1 mL stock solution (1000 µg mL^{-1}) of REM and 3% hydrogen peroxide (H_2O_2) solutions in 10 mL volumetric flask. The solution was then heated at 70 ^{0}C for 30 min and was kept at room temperature to get cooled. The solution sample was analyzed and the densitogram of REM was observed to check degradation.

Thermal degradation

The thermal degradation was performed by exposing the drug sample to REM in the oven at 70 ^{0}C for 2 h. The cooled sample of drug (10 mg) was then dissolved and diluted in a volumetric flask with methanol to a volume of 10 mL.

Photo degradation

The UV light degradation was performed by exposing the drug sample (10 mg) of REM to UV light for 2 h. The sample of drug was then dissolved and diluted in a volumetric flask with methanol to a volume of 10 mL. On TLC plates, all reaction solutions were administered using an applicator µL syringe. Plates were created under optimal chromatographic conditions, and densitograms were taken.

Solution stability

The REM stock solutions were kept at room temperature for 24 h and analysed at 0, 4, 8 and 24 h intervals.

Analysis of marketed formulation

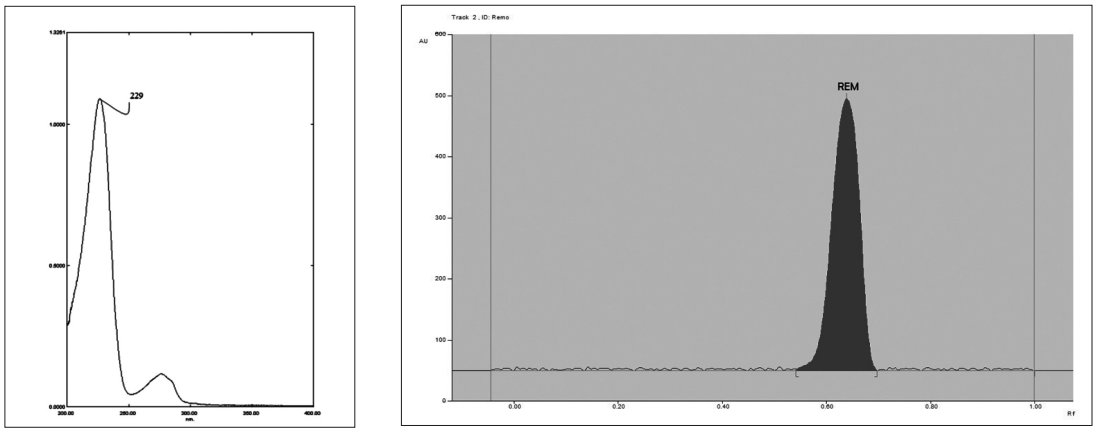
The powdered equivalent weight (50 mg) of 20 tablets was transferred into a 10 mL volumetric flask followed by 5 mL of methanol. The resulting solution was sonicated for 15 minutes. A 0.45 µm Whatman filter paper was used to filter the resulting solution and the volume was adjusted to the mark using methanol (5000 µg mL^{-1}).

Using a sample applicator, 4 µL of sample was applied on the stationary phase which gave 1500 ng $band^{-1}$ concentration of REM. As per optimised chromatographic conditions the stationary phase plates were developed and scanned. The areas were calculated, and the quantification was accomplished by including this value into the regression equation.

RESULTS AND DISCUSSION

The method of analysis of REM was developed and validated by using high performance thin layer chromatography. CAMAG instrument with a Linomat V applicator was used for the analysis of REM. The pre-coated aluminium HPTLC plates with silica gel RP-18 F_{254S} were used as the stationary phase. CAMAG TLC Scanner III was used for densitometric scanning and optimised the mobile phase of ACN: water: ammonia solution (8:2:0.5 V/V/V) (Fig. 2-b) was selected for analysis (the ammonia solution was used as a modifier/tail reducer). The detection wavelength was selected using desitometric measurement in scanning mode in the UV region of 400-200 nm, and the overlaid spectra of REM shows absorption at 229 nm (Fig. 2-a).

The compact band of REM with a retardation factor (R_f) value was found to be 0.72. The saturation time of the chamber was 30 minutes at 25 ± 1^{o}C and 35 ± 5% relative humidity controls. The distance and the time developed were 80 mM and 11 min, respectively, in the ADC2 chamber. The calibration curve in the linearity range was found 500-3000 ng $band^{-1}$ with a linear



(a) (b)

Fig. 2: (a) UV spectra of REM showing absorption maxima at 229 nm

(b) Typical densitogram of REM (R_f 0.72) in acetonitrile: water: ammonia solution (8:2:0.5 V/V/V)

correlation coefficient (r^2) of 0.9995. The final chromatographic condition data shown in (Table I), and the 3D densitogram shown in (Fig. 3).

Table I: Final chromatographic condition for HPTLC

Chromatographic condition	HPTLC
Developing Time	11 min
Temperature	25 °C
Wavelength	229 nm
Linearity range	500-3000 ng band^{-1}
R_f	0.72
Diluent	Methanol
Mobile phase	Acetonitrile: water: ammonia solution (8:2:0.5 V/V/V)

The method's accuracy, precision, and durability (ruggedness) were all confirmed. Recovery experiments at three distinct levels, i.e., 80%, 100%, and 120% were used to validate the method's accuracy. The percentage recovery of REM was found to be in the range of 99.50-102.20% w/w. The method was found to be precise as indicated by the inter-day, intra-day, and repeatability analysis; showing %RSD. The LOD and LOQ of REM were found to be 3.84 and 39.44 ng band^{-1}, respectively. The results did not show any statistical difference between operators, suggesting that the method developed was rugged. The results of the developed method are shown in (Table II).

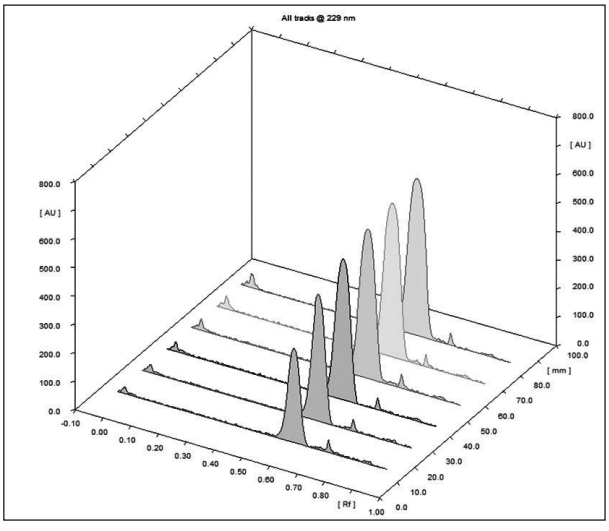


Fig. 3: Overlaid 3D densitogram of REM

Table II: Summary of validation parameters

Parameter	Result
Linearity	500-3000 ng band^{-1}
Correlation coefficient (r^2)	0.9975
LOD	3.84 ng band^{-1}
LOQ	39.44 ng band^{-1}
Accuracy (%)	98.67-100.13
% Recovery (n=3)	98.22 ±100.22 w/w
Intermediate precision (%RSD)	
Intra-day(n = 3)	0.50-0.77
Inter-day (n = 3)	0.43-0.82
Robustness (%RSD)	0.39-0.63

n = No of determinations

Forced degradation study

The forced degradation studies were performed by using different parameters as per ICH Q1A (R2) and Q1B guidelines[15]. The current study was more focused on acid degradation as it was observed that REM undergoes further degradation with time in acidic conditions. The base hydrolysis using 0.1 N NaOH suggests that the drug undergoes degradation rapidly at room temperature (25 ± 2 °C) for 30 min. The repetition (5 min) after neutralisation shows the peak of drug and degradant at R_f 0.43 and 0.72 in the densitogram (Fig. 4).

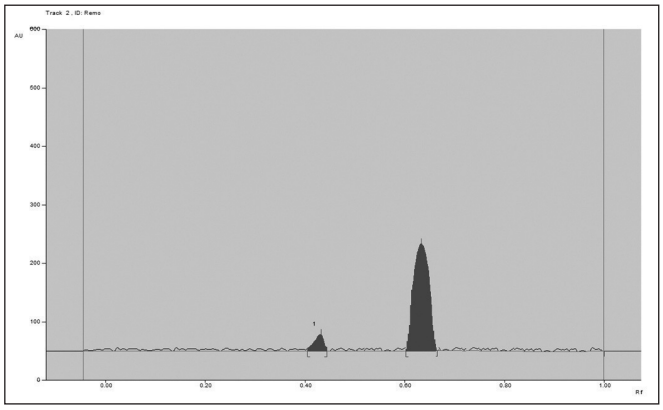
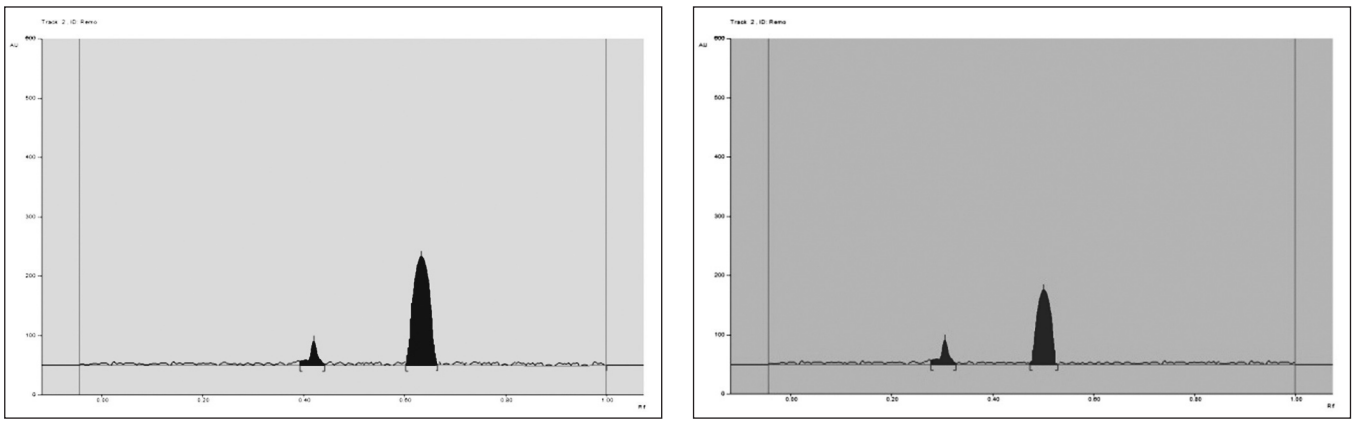


Fig. 4: Base degradation of REM (0.1 N NaOH)

The acid hydrolysis data of REM using 0.1 N HCl at room temperature suggests that the drug slowly undergoes degradation, while heating the drug at 70 °C slightly increases the degradation rate. Initially, at 30 min, REM shows resolution of two peaks, drug and degradant, at R_f of 0.42 and 0.72, respectively, in the densitogram (Fig. 5-a).



(a) (b)

Fig. 5: Acid degradation of REM

(a) Acid degradation of REM at 30 min showing peak of drug at R_f 0.72 and degradant at (D1) R_f 0.42

(b) Acid degradation of REM at 36 h showing peak of degradant (D3) at R_f 0.29 and (D2) at R_f 0.51

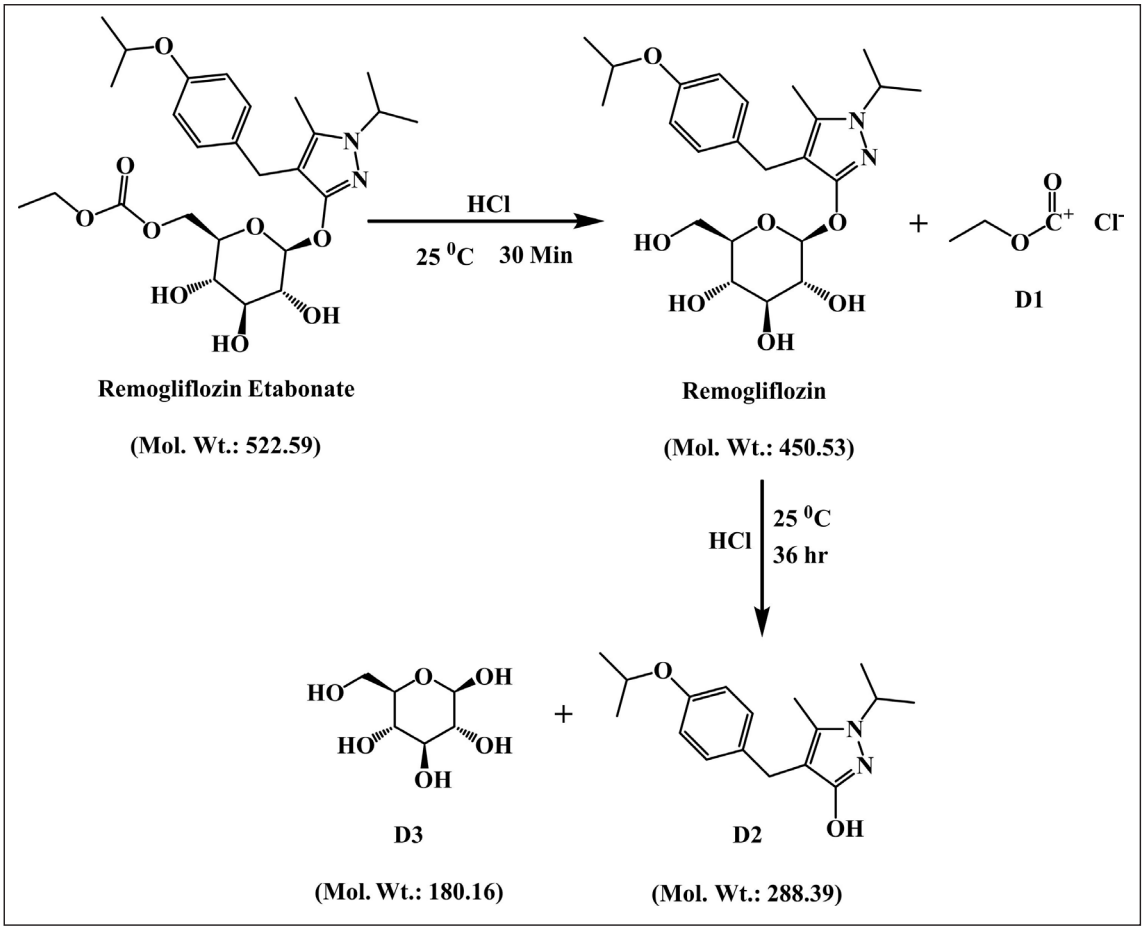


Fig. 6: Degradation pathway of REM; Acid degradation at 30 min (RT) of REM (pro drug) provide active constituent with single degradant (D1); Acid degradation at 36 h (RT) active form provide (D2) and (D3) as separate degradant

Further, extending the acid degradation time of REM to 36 h, at room temperature, another two degradants (D2 and D3) are found to be resolved (pharmacologically inactive) at R_f 0.29 and 0.51. The oxidative degradation study using hydrogen peroxide showed that REM undergoes degradation at R_f 0.46 (Fig. 5-b). Stability was observed for REM in dry heat and photo degradation. The data on forced degradation is summarised in Table III.

Table III: Summary of stress condition for degradation

Type of degradation	Stress condition	Sampling time	R_f value of degradant
Acid degradation	1.1 N HCl, 25 °C	30 min	0.42
Acid degradation	1.1 N HCl, 25 °C	36 h	0.29 and 0.51
Alkali degradation	0.1 N NaOH, 25 °C	30 min	0.43
Oxidative degradation	3% H_2O_2, 25 °C	30 min	0.46
Photolytic degradation	UV light	24 h	-

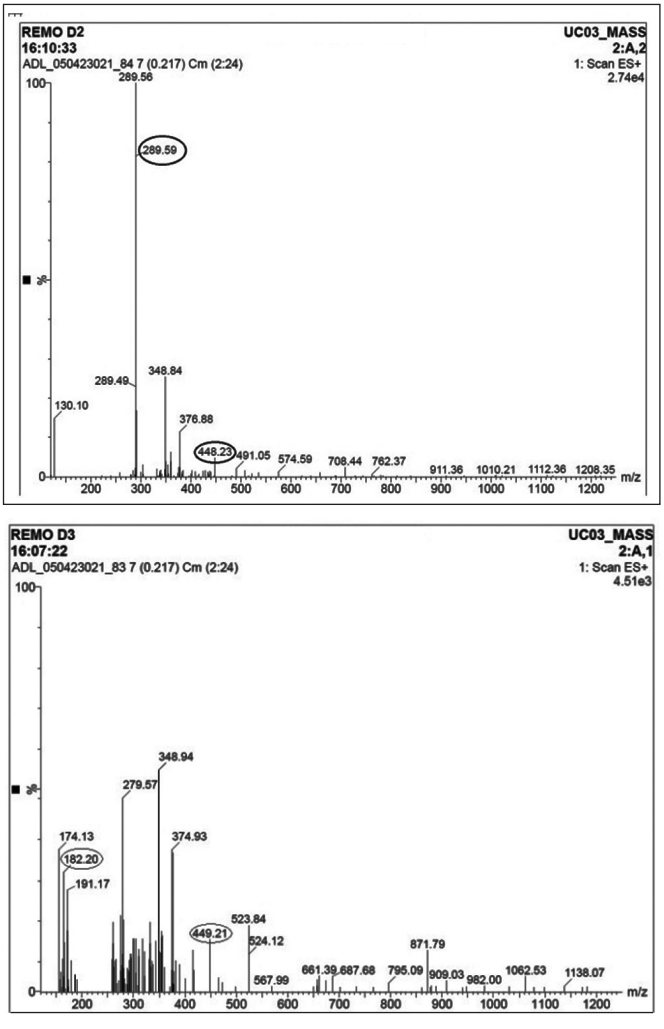


Fig. 7: Mass spectra of degradant D2 and D3 showing m/z at 289 (M+1) for D2 and 182 (M+2) for D3 (m/z of D2 was observed same as molecular weight of inactive GSK1132678 as shown in Fig. 1)

DISCUSSION

The extensive literature survey reveals the selection of an optimised mobile phase on the basis of the polarity of the solvent. Initially, many solvents were tried in selective fractions for the resolution of REM. A mixture of ACN: water: ammonia solution (8:2:0.5 V/V/V) was selected as the optimised mobile phase, showing the final band at R_f 0.72. The linearity of the developed method was found to be 500-3000 ng band^{-1}, while the accuracy data was found to be 100.13%, suggesting the accuracy of the method. Moreover, all the validation related data like %RSD, LOD, LOQ and robustness was observed under specifications. The forced degradation studies were carried to find out the degradation pathway of REM. From all the stressed conditions, REM is found to be susceptible to acid, alkali, and oxidative degradation, while remaining stable at photolytic and under exposure to dry heat. The exposure of REM to acidic conditions for a long time (36 h) showed two different degradant products (other than the drug and the initial degradant at 30 min). The initial degradant (D1) as per (Fig. 5a) with the peak of drug was observed at R_f 0.42, while extending the acid degradation to 36 h showed the peaks D2 and D3 at R_f 0.29 and 0.51, respectively (Fig. 5b). Both the degradants D2 and D3 were then characterised by using mass spectrometry (Fig. 7) for the determination of the structures of these degradants and the probable pathway for degradation.

The possible degradation pathway is depicted in (Fig. 6), which states that the initial degradant (D1) was obtained within 30 min when the drug was hydrolyzed with acid. Further, by increasing the time of hydrolysis for 36 h, REM gets converted into two different inactive metabolites, D2 and D3. The prediction of the structure of degradants was not possible using single HPTLC spectroscopy, and hence mass spectroscopy was used to confirm the structure of the degradant. The mass spectra shows an m/z value of 289.39 (M+1) for D2 and 182 (M+2) for D3, having a molecular weight of 288.39 gm mol^{-1} and 180.16 gm mol^{-1} respectively (Fig. 7).

On comparing the predicted mass spectra with the metabolic pathway of REM (Fig. 1), the inactive product GSK1132678[4] was observed with a molecular weight of (288.39 g mol^{-1}), which was obtained by the oxidative process of the hydroxyl group of glucose result to form glucuronic acid in human liver cytosol in the presence of UDP-glucose dehydrogenase[4]. It indicates that REM forms an active degradant when in short contact with acid and long-term contact forms an inactive degradant.

CONCLUSION

A novel, simple and precise RP-HPTLC method for determining REM in bulk and pharmaceutical dosage form has been successfully developed and validated. The validated method was quick, accurate, reliable and rugged. It is also revealed that the system is accurate and sensitive. The percent recovery in formulation shows that the excipients used in the formulation have no effect on the result. The forced degradation studies reveal that the acid degradation of REM delivers active metabolites (in a short period) and inactive metabolites (in a long period) with acid as predicted via reported metabolism. Hence, the proposed method can be used to estimate REM in bulk and pharmaceutical formulations on a regular basis.

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