International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 15, Issue 8, 2023

Original Article

# HYDROLYTIC DEGRADATION STUDY OF ROXADUSTAT BY RP-HPLC AND HPTLC

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*Received: 18 May 2023, Revised and Accepted: 13 Jun 2023*

**ABSTRACT**

**Objective**: Simple, rapid RP-HPLC and HPTLC methods have been developed in order to study the degradation of Roxadustat under various stress conditions. The Kinetics of hydrolytic degradation is studied.

**Methods**: Optimum separation of Roxadustat and its degradation products was achieved using the following conditions in HPLC, Agilent eclipse XDB-C8 (150×4.6 mm) column, the mobile phase was composed of methanol: phosphate buffer (pH 5, 0.05 M) (70:30 *v/v*) with UV detection at 262 nm. The flow rate was at 1.0 ml/min. The RT was 4.6±0.02 min. HPTLC work for Roxadustat was performed on Aluminium plates precoated with silica gel 60 $F_{254}$, (10 cm × 10 cm with 250 µm layer thickness). The mobile phase was composed of Toulene: Ethyl Acetate: Glacial acetic acid (5:5:0.5 *v/v/v*) and then scanned. The system was found to give a compact spot for Roxadustat (Rf value of 0.58±0.02).

**Results**: In HPLC the calibration curves plotted were found to be linear over the concentration range of 2.5-25µg/ml, with a correlation coefficient of $R^2$=0.9994. In HPTLC the calibration curves plotted were found to be linear over the concentration range of 500-2500 ng/band, with a regression coefficient of $R^2$=0.9957. The analytical performance of the proposed methods was validated as per ICH Q2 (R1) guidelines. The degradant peaks were well resolved from the Roxadustat peak. Significant degradation was observed in acid hydrolysis, alkali hydrolysis, and oxidative degradation. The drug is relatively stable towards photolysis, neutral hydrolysis, and thermal conditions.

**Conclusion**: In the current work, simple RP-HPLC and HPTLC analytical methods for the determination of Roxadustat in the presence of its degradation products have been developed. The information presented herein could be very useful while developing formulation procedures to prevent hydrolytic degradation. It can be used as a routine quality control test.

**Keywords**: RP-HPLC, HPTLC, Roxadustat, Hydrolytic degradation, Stress conditions


DOI: https://dx.doi.org/10.22159/ijpps.2023v15i8.48355. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps.

## INTRODUCTION

Roxadustat is used in the treatment of anemia. It is an oral inhibitor of hypoxia-inducible factor (HIF) prolyl hydroxylase, which promotes erythropoiesis and controls iron metabolism [1, 3]. The chemical name of Roxadustat is 2-[(4-hydroxy-1-methyl-7-phenoxyisoquinoline-3-carbonyl) amino] acetic acid. Its molecular formula is $C_{19}H_{16}N_2O_5$ and its molecular weight is 352.3 g/mol, respectively. The chemical structure of Roxadustat is shown in fig. 1.

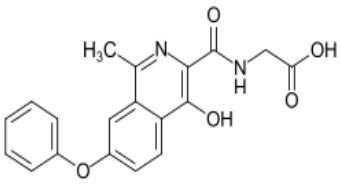



**Fig. 1: Structure of roxadustat**

An important function of the kidneys is the production of erythropoietin, a hormone that stimulates the production of red blood cells [4]. In chronic kidney disease (CKD), the kidneys are not functioning at capacity and there can be a disruption in the production of erythropoietin that can lead to anemia [5, 6]. Anemia is a chronic kidney disease complication associated with increased risks of death and complications [7]. It decreases endogenous erythropoietin production, functional iron deficiency, and inflammation with increased hepcidin levels, among others [8].

Roxadustat stabilizes HIF-α (hypoxia-inducible factor) subunits, which increases HIF transcriptional activity. The increased transcriptional activity produces functional activation of early-response target genes that encode proteins like erythropoietin, erythropoietin receptor, heme biosynthesis enzymes, and proteins that facilitate iron uptake and transport. For the treatment of anemia in patients with chronic kidney diseases who are dependent on dialysis, non-dependent on dialysis, and myelodysplastic syndromes, Roxadustat is a highly effective first-in-class HIF-PHD (hypoxia-inducible factor-prolyl hydroxylase inhibitor) [9, 10].

As per the literature survey for Roxadustat Liquid chromatography-tandem mass spectrometry, UPLC–MS method and UV method were reported, but no stability indicating HPLC and HPTLC method is reported [11, 13].

The present work describes the simple, accurate chromatographic analytical methods for determining Roxadustat.

## MATERIALS AND METHODS

### Chemical and reagents

Roxadustat was kindly gifted by industry. Other chemicals and reagents like Methanol (HPLC grade), Potassium dihydrogen phosphate (AR grade), Dimethyl sulfoxide (DMSO), Glacial Acetic Acid (AR grade), Toulene (AR grade), Ethyl Acetate (AR grade), Hydrochloric acid (AR grade), Sodium Hydroxide (AR grade), and Hydrogen Peroxide (AR grade) were procured from LOBA CHEMIE PVT. LTD., Mumbai.

### Instrumentation and chromatographic conditions

Instruments used in this method were HPTLC system (CAMAG) comprising of TLC Scanner III, Linomat 5 applicator, Software [win CATS (version 1.4.3)], Microliter syringes [Hamilton (100 µl)], TLC plates (Merck's aluminum TLC plate precoated with silica gel 60 $F_{254}$), Twin trough glass chamber. The HPLC was done on HPLC-PDA

(Photo Diode Array Detector, (Make-JASCO, Model-Pump PU-2080) and Detector MD 2010. A photostability study was performed in a photostability chamber (Make NEWTRONIC Model-NEC103RSPI). Other instruments used are UV-Visible spectrophotometer (SHIMADZU Model-UV 1780), Electronic balance (Make SHIMADZU Model ATX-224R), Sonicator (PRAMA Model SM15 US), Hot air oven (BIOMEDICA 24*24*24*).

**Preparation of stock and standard working solution**

**HPLC and HPTLC**

An accurately weighed 25 mg of Roxadustat was transferred into a 25 ml volumetric flask and added 0.5 ml DMSO and shaken well until it gets dissolved. After that volume was made up with methanol to get standard stock solutions of Roxadustat (1000 µg/ml). From this, 1 ml was taken in a 10 ml volumetric flask and the volume was made up with methanol to get 100µg/ml of Roxadustat. For HPTLC we used 100 µg/ml as a working standard and for HPLC, six standard solutions of different concentrations (2.5, 5, 10, 15, 20, 25 µg/ml) were prepared with the mobile phase.

**Detection wavelength**

A solution of 20 µg/ml was prepared and scanned over 200-400 nm using a UV–Spectrophotometer. The maximum absorbance was shown at 262 nm. The spectrum is shown in fig. 2.

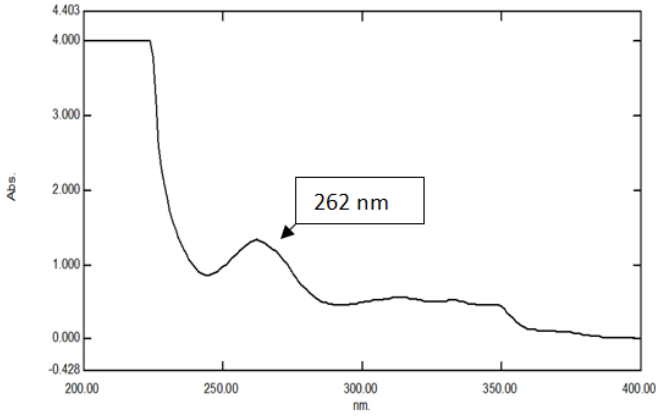



**Fig. 2: UV spectrum of roxadustat (20 µg/ml)**

**Chromatographic conditions**

**HPLC**

The method was developed on the Agilent eclipse XDB-C8 (150×4.6 mm) column. The mobile phase was composed of methanol: phosphate buffer (pH 5, 0.05 M) (70:30 *v/v*) with UV detection at 262 nm. The flow rate was 1 ml/min. The mobile phase was filtered through a 0.45 µmembrane filter and sonicated for 10 min. The standard chromatogram of Roxadustat (10 µg/ml) is shown in fig. 3.

**HPTLC**

Chromatographic separation of roxadustat drug was performed on aluminium plates precoated with silica gel 60 $F_{254}$, (10 cm × 10 cm with 250 µm layer thickness). Samples were applied on the plate as a band of 6 mm width using a 100 µl syringe with a Linomat applicator. The mobile phase was composed of Toulene: Ethyl Acetate: Glacial acetic acid (5:5:0.5*v/v/v*). The twin trough glass chamber10 cm × 10 cm was used for linear ascending development of the TLC plate with 15 min saturation conditions; migration distance was 70 mm. Densitometric scanning was performed at 262 nm, operated by CAMAG (win CAT SOFTWARE 1.4.2), and slit dimensions were 5 x 0.45 mm. The standard Densitogram of roxadustat (1000 ng/band) is shown in fig. 4.

**Forced degradation study**

To develop a stability-indicating method, forced degradation studies were carried out according to ICH Q1A (R2) guideline [13]. A literature for optimization of stress conditions was preferred [14, 15]. The drug was exposed to various stress conditions for varying periods of time and using various strengths of reagents. The drug was exposed to acid/alkaline hydrolysis, oxidation, neutral, photolytic, and thermal degradation conditions. stress conditions were optimized to achieve degradation of about 10-30 %.

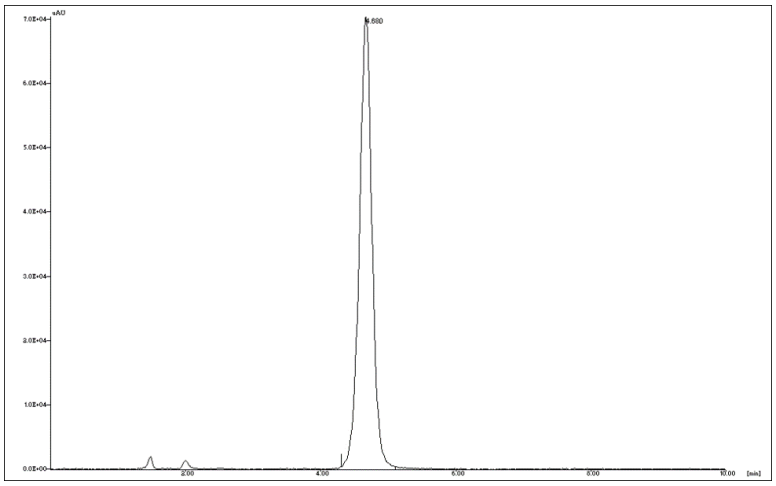


**Fig. 3: Chromatogram of roxadustat (10 µg/ml);RT=4.6±0.02 min**

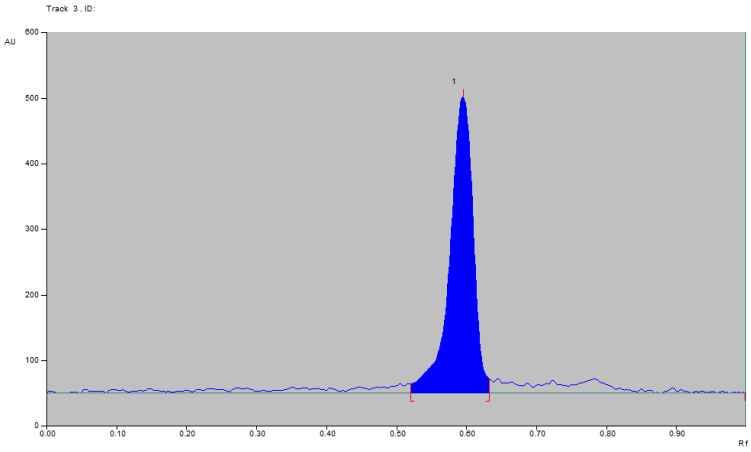



**Fig. 4: Densitogram of roxadustat (1000 ng/band); Rf=0.58±0.02**

**Acid hydrolysis**

**HPLC**

For sample preparation, 1 ml of standard stock solution (1000 µg/ml) was mixed with 1 ml of 1 N HCL in a 10 ml volumetric flask and the volume was made up with methanol. The solution was placed at room temperature for about 2 h. From this solution, 1 ml was taken and neutralized with 0.1 ml of $^{1}$N NaOH, and the volume was made up to 10 ml with mobile phase to obtain 10 µg/ml. The resulting solution was injected into the system.

**HPTLC**

From the standard stock solution (1000 µg/ml) 1 ml was mixed with 1 ml of $^{1}$N HCl and the volume was made up to 10 ml with methanol. The solution was kept for 2 h at room temperature. The resultant solution of 100 µg/ml was applied to the TLC plate and developed using an optimized mobile phase.

**Alkali hydrolysis**

**HPLC**

From the standard stock solution (1000µg/ml) 1 ml was mixed with 1 ml of 1 N NaOH in a 10 ml volumetric flask and the volume was made up with methanol. The solution was placed at room temperature for about 2 h. From this solution, 1 ml was taken and neutralized with 0.1 ml of $^{1}$N HCL, and the volume was made up to 10 ml with mobile phase to obtain 10 µg/ml. The resulting solution was injected into the system.

**HPTLC**

For sample preparation, 1 ml of the standard stock solution (1000 µg/ml) was mixed with 1 ml of 1 NaOH and the volume was made up to 10 ml with methanol. The solution was kept for 2 h at room temperature. The resultant solution of 100 µg/ml was applied to the TLC plate and developed using an optimized mobile phase.

**Oxidative degradation**

**HPLC**

For sample preparation, 1 ml of the standard solution (100 µg/ml) and 1 ml of 30% *v/v*$H_2O_2$ were transferred to a 10 ml volumetric flask and the volume was made with the mobile phase to obtain 10 µg/ml. The solution was kept at room temperature for about 4 h. The resulting solution of 10 µg/ml was injected into the system.

**HPTLC**

From the standard stock solution (1000µg/ml) 1 ml was mixed with 1 ml of 30%*v/v*$H_2O_2$andthe volume was made up to 10 ml with methanol. The solution was kept at room temperature for 4h. The resultant solution of 100 µg/ml was applied to the TLC plate and developed using an optimized mobile phase.

**Neutral degradation**

**HPLC**

For sample preparation, 5 ml from the standard stock solution (1000 µg/ml) and 5 ml of water were transferred to a 50 ml volumetric flask and the volume was made with methanol and refluxed for 6 h at 80 °C and cooled at room temperature. From this taken 1 ml and diluted up to 10 ml with the mobile phase to obtain 10 µg/ml. The resulting solution of 10 µg/ml was injected into the system.

**HPTLC**

After refluxing the resultant solution of 100 µg/ml was applied to the TLC plate and developed using an optimized mobile phase.

**Thermal degradation**

**HPLC**

The thermal degradation was carried out by placing the drug in a solid state in an oven at 80 °C for 8 h. A sample was taken from the oven, cooled to room temperature, weighed, and diluted in the mobile phase to provide a final concentration of 10 µg/ml of Roxadustat, which was then injected into HPLC and evaluated under optimum chromatographic conditions.

**HPTLC**

The drug exposed to thermal degradation was appropriately weighed and diluted in methanol to provide a final concentration of 100 µg/ml. The resultant solution of 100 µg/ml was applied to the TLC plate and developed using an optimized mobile phase.

**Photolytic degradation**

**HPLC**

Accurately weighed 50 mg drug and transferred into a clean petri dish and exposed to UV light till exposure 200-watt h/m2 and to white cool fluorescent light up to 1.2 million lux h. After completion of the required illumination, the sample was removed. Appropriately weighed and diluted in the mobile phase to obtain 10µg/ml. The resultant solution was injected into HPLC and analyzed under optimized chromatographic conditions.

**HPTLC**

The drug exposed to photolytic degradation was appropriately weighed and diluted in methanol to obtain 100µg/ml. The resultant solution was applied to the TLC plate and developed using an optimized mobile phase.

**Method validation**

The HPLC and HPTLC methods for Roxadustat were validated as per the ICH guidelines ICH Q2(R1) in terms of linearity, range, assay, accuracy, precision, limit of detection, limit of quantitation, and

robustness [16]. A literature methods were studied for procedural details of validation parameters [17, 18].

**Linearity and range**

**HPLC**

The dilutions were prepared from a standard stock solution of Roxadustat (1000 µg/ml). The linearity was observed over the range of 2.5-25 µg/ml. The calibration curve was made using six standard solutions of different concentrations (2.5, 5, 10, 15, 20, and 25 µg/ml). The standard solutions were prepared by diluting an appropriate volume of stock solution with the mobile phase. The procedure was repeated 5 times to get the linear regression equation. The values were plotted as concentration against peak area to obtain a calibration curve. The pattern of the residual plot was also evaluated to further validate the linearity.

**HPTLC**

The standard solutions were prepared by diluting an appropriate volume of stock solution with the methanol. The linearity was observed over the range of 500-2500 ng/band. The procedure was repeated 5 times to get the linear regression equation. The calibration curve was made using five standard solutions of different concentrations (500, 1000, 1500, 2000 and 2500 ng/band). The values were plotted as the amount of drug spotted (ng/band) against the peak area to obtain a calibration curve. The pattern of the residual plot was also evaluated to further validate the linearity.

**Precision**

**HPLC**

The precision was performed both for intraday (repeatability) and interday (intermediate) studies. For intraday, injecting 6 replicates of 2.5 µg/ml concentration within the same day, and for interday precision, the procedure was repeated on three consecutive days.

**HPTLC**

The precision study was performed as intraday (repeatability) precision and interday (intermediate) precision. Intraday precision was performed by analyzing 500 ng/band of Roxadustat, as six replicates on the same day at different intervals. For interday precision, the procedure was repeated on three consecutive days.

**Assay**

**HPLC and HPTLC**

The assay of Roxadustat was done on the spiked blend, due to the unavailability of its marketed preparation in the Indian market. For the preparation of the spiked blend, weighed 1000 mg starch and 1000 mg lactose was mixed in the mortar pestle. Then 500 mg of Roxadustat was mixed with the above excipients. From this spiked blend, 50 mg of the blend (equivalent to 10 mg of the drug) was accurately weighed and transferred in a 10 ml volumetric flask. Add 0.5 ml of DMSO and shake well till it gets dissolved and the test tube was filled with 5 ml methanol. Shake well and sonicated for 10 min. The solution was centrifuged and filtered through Whatman filter paper. This filtrate was transferred to a 10 ml volumetric flask and made up the volume to 10 ml with methanol. From this taken 1 ml was in a 10 ml volumetric flask and diluted up to 10 ml with methanol. From the resulting solution, 1 ml was transferred in a 10 ml volumetric flask and diluted up to 10 ml with the mobile phase to get 10 µg/ml of roxadustat. For HPLC the resulting solution of 10 µg/ml was injected into the system at room temperature. And for HPTLC the concentration of 100 µg/ml was applied to the TLC plate and developed using an optimized mobile phase. The amount of Roxadustat was calculated using the linearity regression equation.

**Accuracy**

**HPLC and HPTLC**

The accuracy was evaluated as a percentage of recovery from the spiked samples at three concentration levels. The recovery study was done by performing the standard addition method at 80%, 100%, and 120% levels. The standard drug has been added the to assay solution at three levels. For HPLC the basic concentration of the sample chosen was 10µg/ml and for HPTLC the basic concentration of the sample chosen was 1000 ng/band respectively. The percentage recovery was calculated by extrapolation from the linear equation.

**Robustness**

**HPLC**

The robustness was examined by evaluating the influence of small variations in different conditions such as mobile phase ratio2 ml v/v), flow rate (±0.05 ml/min), and pH of buffer (±0.2). The average value of % RSD for the determination of Roxadustat less than 2% confirmed the robustness of the method.

**HPTLC**

The robustness was examined by evaluating the influence of small variations in different conditions such as saturation time (±5 min), wavelength (±2 nm), mobile phase ratio (±0.2 ml *v/v*), time for an application to development and time for development to scanning. The average value of % RSD for the determination of Roxadustat less than 2% indicated the robustness of the method.

**LOD and LOQ**

The LOD and LOQ were calculated by using the equation LOD = 3.3 X SD/S and LOQ = 10 X SD/S; where, 'SD' is the standard deviation of response at the lowest concentration, and 'S' is the slope of the calibration curve.

**RESULTS AND DISCUSSION**

**Forced degradation studies**

**HPLC**

The Roxadustat was found degraded under acid hydrolysis, alkali hydrolysis, and oxidation conditions, and degradant peaks were observed under acid, and alkaline conditions. Degradation products were completely resolved from the parent compound. The summary of results is presented in table 1 and the chromatogram of roxadustat subjected to acid, and alkaline stress conditions is shown in fig. 9 and fig. 10, respectively.

In acidic hydrolysis, degradant peaks were obtained at an RT of 12.5 min, and in basic hydrolysis, degradant peaks were obtained at an RT of 3.7 and 4.2 min well resolved from the drug peak. Overall Roxadustat degradant peaks were observed at RT of 12.5, 3.7, and 4.2 min, respectively.

**HPTLC**

In acidic hydrolysis, the degradant peak was obtained at Rf of 0.70. The densitogram of acid hydrolysis is shown in fig. 5 and a spectral overlay for acid degradation product and the standard drug is shown in fig. 6 respectively. In basic hydrolysis, a degradant peak was obtained at Rf of 0.54. The densitogram of alkali hydrolysis is shown in fig. 7 and a spectral overlay for alkali degradation product and the standard drug is shown in fig. 8 respectively.

As per the literature survey for Roxadustat Liquid chromatography-tandem mass spectrometry, UPLC–MS method and UV method were reported but no stability indicating HPLC and HPTLC method is reported [11, 13].

**Degradation kinetic study**

**HPLC**

The order of degradation reaction was established by different methods like substitution, graphical, and Half-life method for acid and base hydrolysis (1 N) by determining recovery at different time intervals from 0-5 h shown in table 2 [19].

The chromatograms of acid hydrolysis with drug product from 0-5 h are shown in fig. 9.

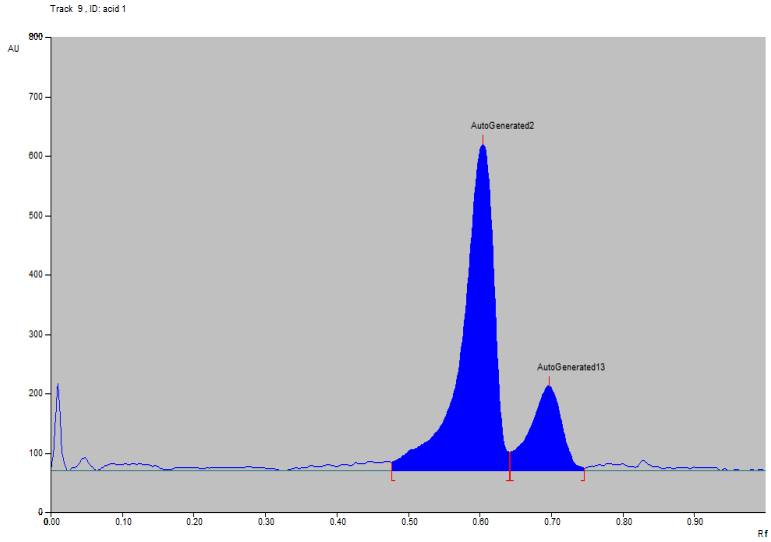



**Fig. 5: Densitogram of acid hydrolysis after 2 h (Rf: 0.60 and degradation product Rf: 0.70)**

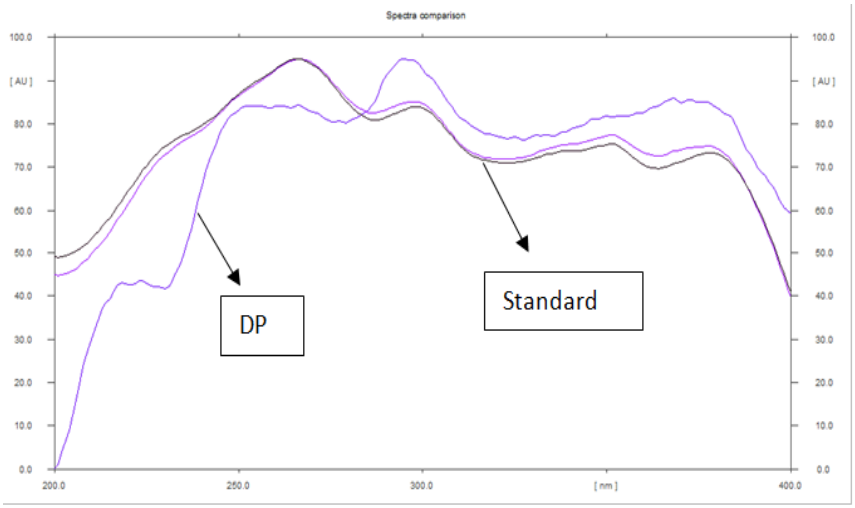



**Fig. 6: Spectral overlay for acid degradation product (Rf: 0.70) and standard drug (Rf: 0.60)**

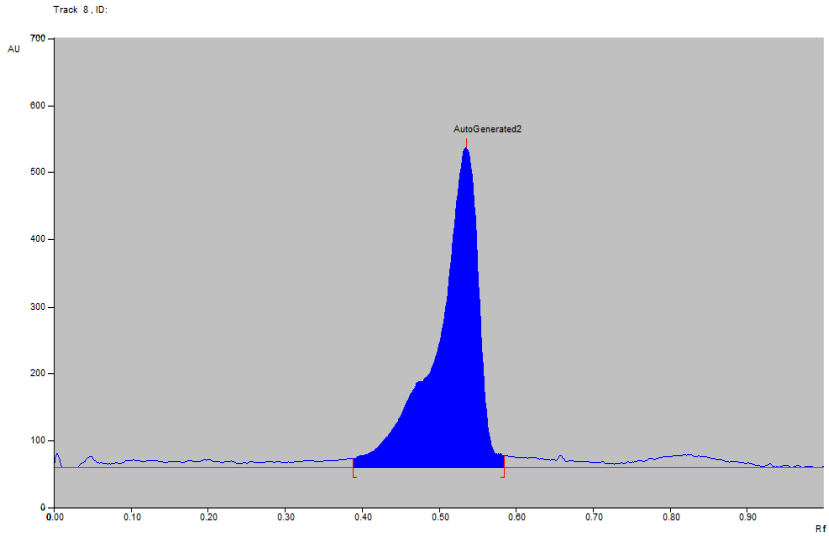



**Fig. 7: Densitogram of alkali hydrolysis after 2 h with drug product (Rf: 0.54)**

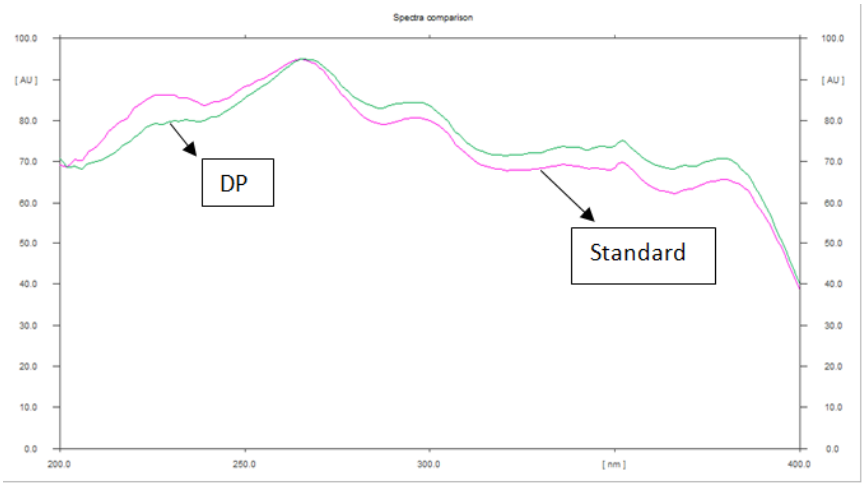
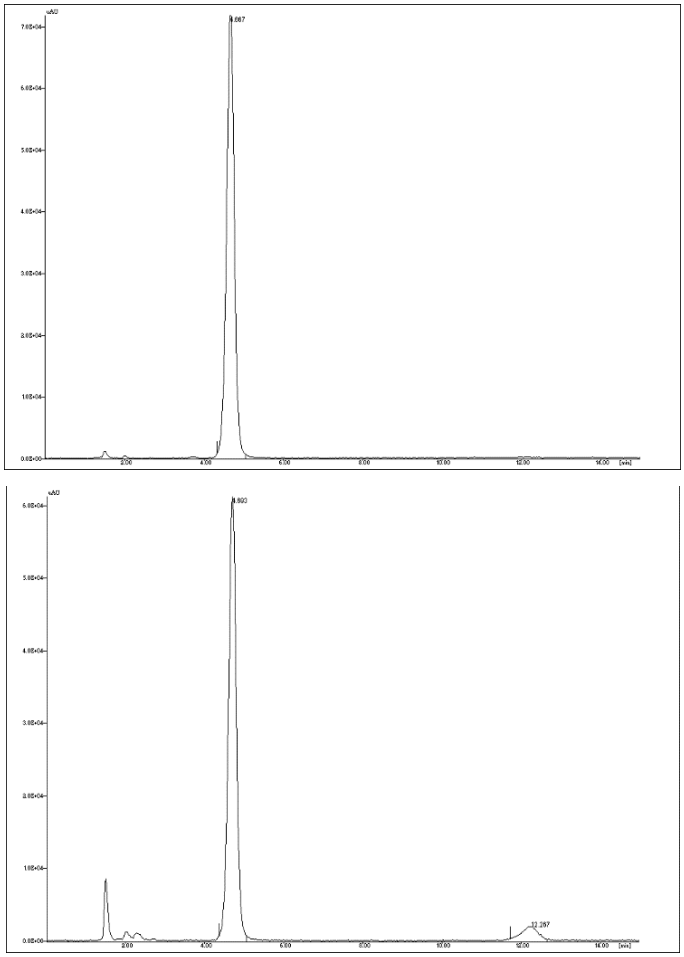
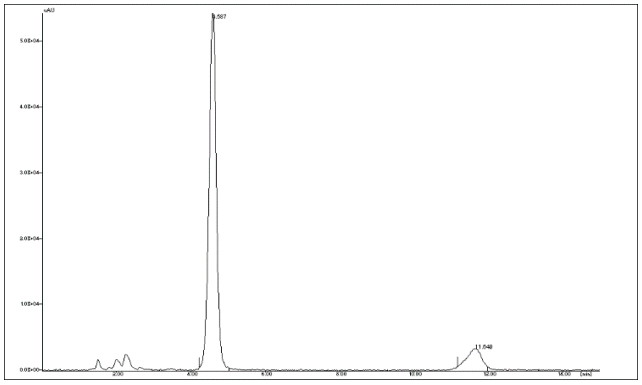



**Fig. 8: Spectral overlay for alkali degradation product (Rf: 0.54) and standard drug (Rf: 0.58)**

**Table 1: Results of forced degradation studies**

| S. No. | Stress type | Stress condition | HPLC % recovery | HPTLC % recovery |
|---|---|---|---|---|
| 1 | Acidic hydrolytic | 1N HCL at RT for 2 h | 74.03 | 89.79 |
| 2 | Alkali hydrolytic | 1N NaOH at RT for 2 h | 71.55 | -- |
| 3 | Oxidative | 30% $H_2O_2$ for 4 h | 83.15 | 78.31 |
| 4 | Neutral | 6 h reflux at 80 °C | 93.92 | 97.04 |
| 5 | Thermal | 80 °C for 8 h | 93.41 | 97.60 |
| 6 | Photostability: 1) UV | 200-watt h/m2 | 98.06 | 98.96 |
| | 2)cool white Fluorescent light | 1.2 million lux h | 97.18 | 96.09 |





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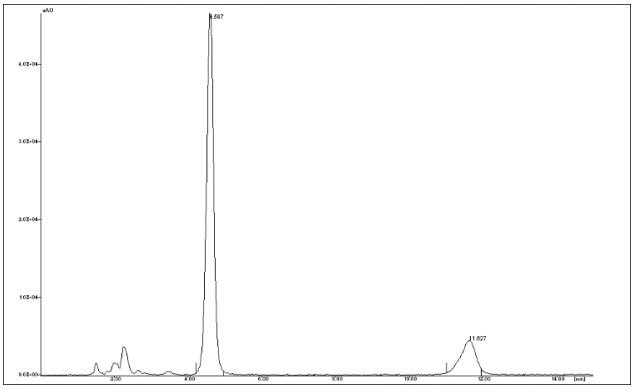

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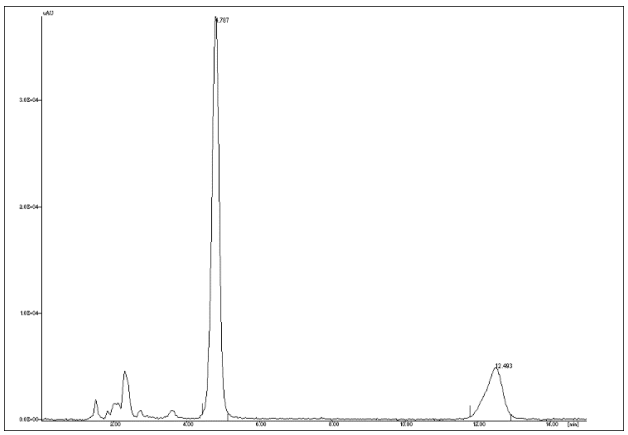

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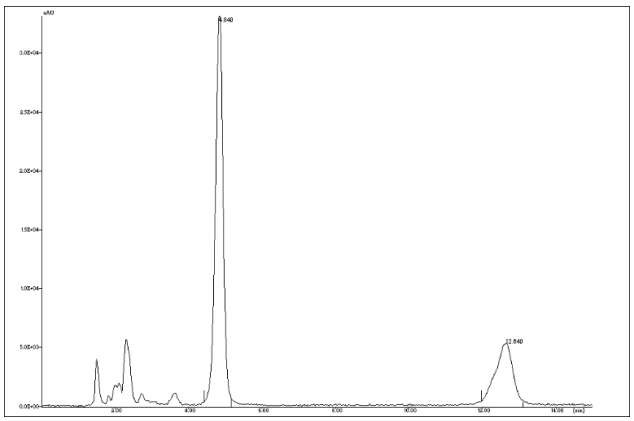
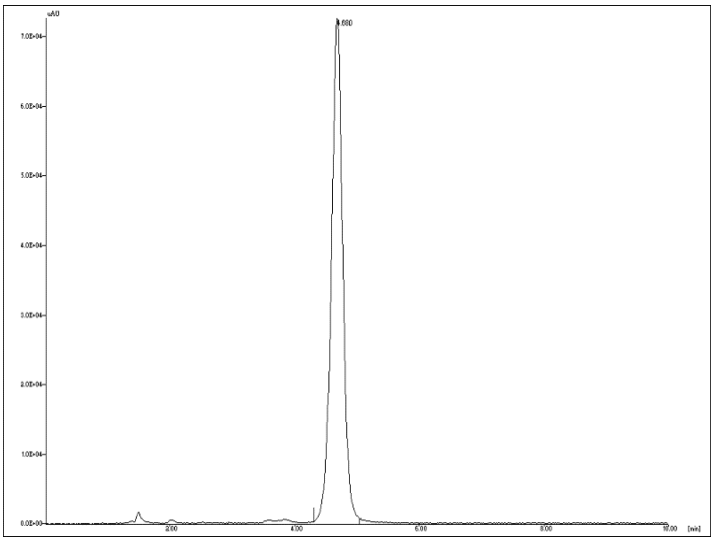


**d**

**Fig. 9: Chromatograms showing Acidic degradation with drug product peak at RT 12.4 min: chromatogram in the time (a) 0 min(b) 1 h (c) 2 h (d) 3 h (e) 4 h (f) 5 h**

The chromatograms of alkali hydrolysis with drug product from 0-5 h are shown in fig. 10.



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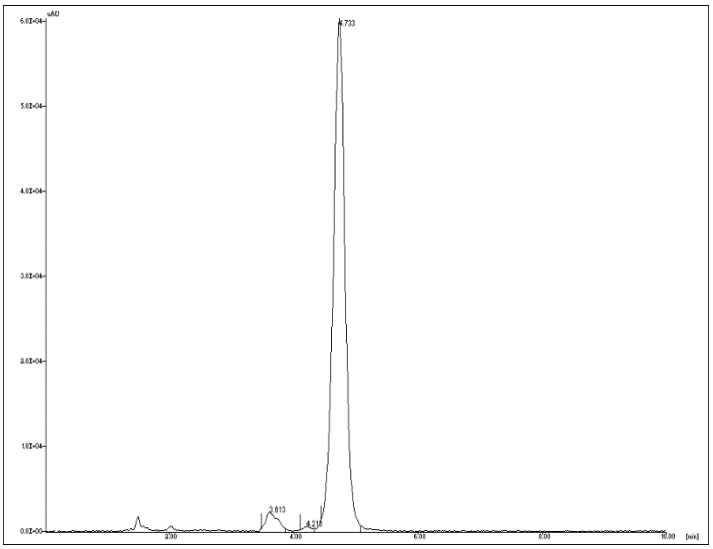

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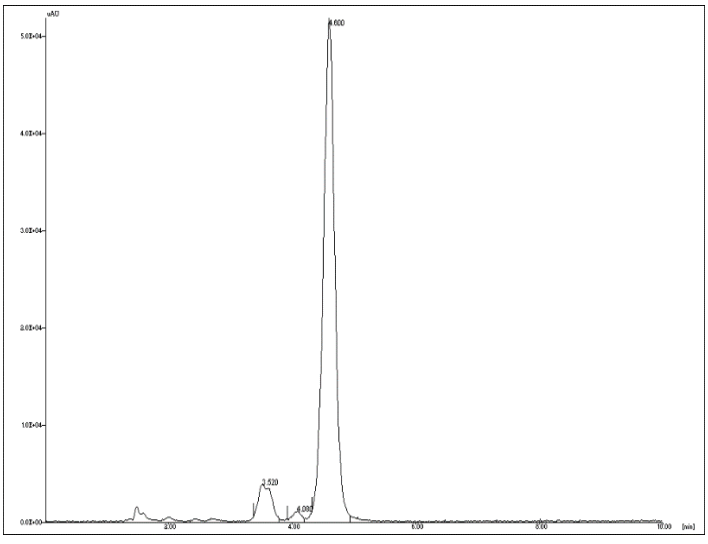

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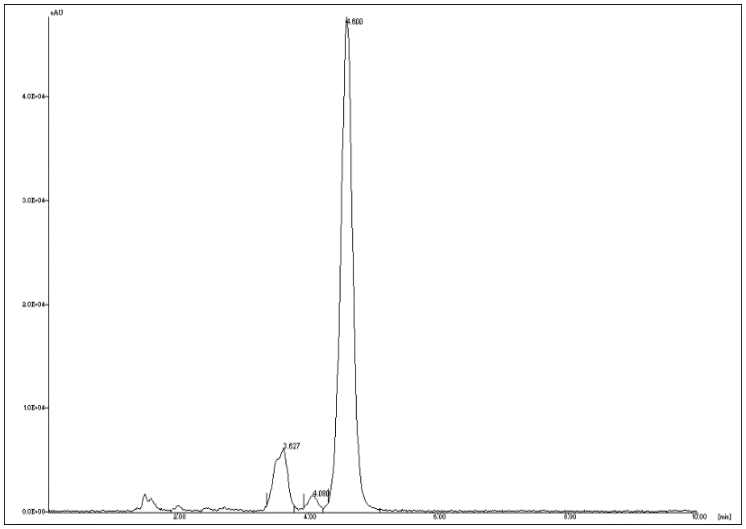
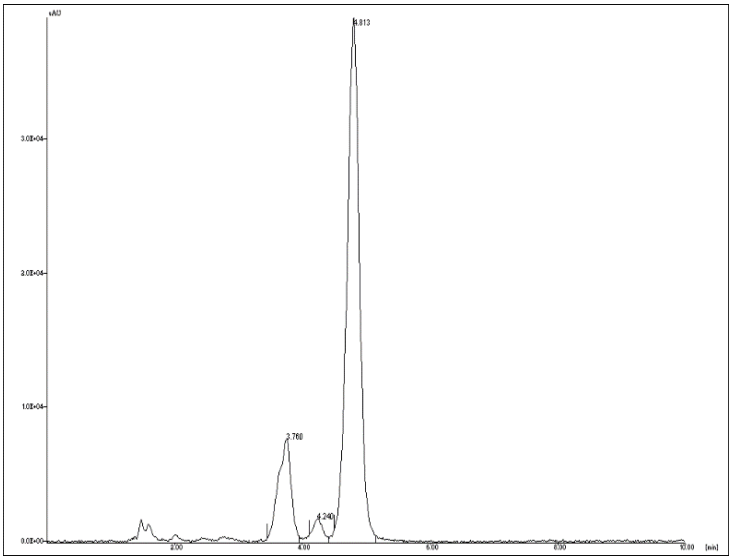
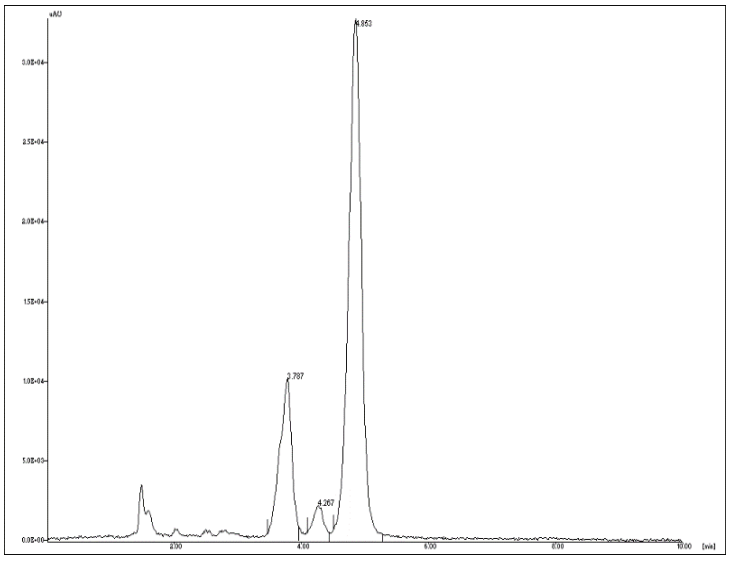



**d**




**e**




**f**

**Fig. 10: Chromatograms showing alkali degradation with drug product peak at RT 3.7, 4.3 min: chromatogram in the time (a) 0 min (b) 1 h (c) 2 h (d) 3 h (e) 4 h (f) 5 h**

**a) Substitution method**

For the substitution method, the following formula was used to determine the rate constant K,

$$K=\frac{2.303}{t}\log\frac{co}{ct}$$

Where, K= rate constant, t= time in h, co= initial concentration, ct=concentration at time t

Since the value of K is fairly constant for both acid and base hydrolysis shown in table 2. Thus, acid and base follow first-order kinetics.

**b) Graphical method**

When the Log of remaining concentration was plotted against time, a straight line was obtained for both acid and base hydrolysis. A straight line confirms a first-order reaction. Thus, acid and base follow a first-order reaction. The Kinetic curves for the hydrolytic degradation reaction are shown in fig. 11.

**c) Half-life method**

The half-life is calculated by the following formula,

$$t_{1/2}=\frac{0.693}{K}$$

Where, K= rate constant, $t_{1/2}$=half-life

The half-life is found to be independent of initial concentration for both acid and base hydrolysis, shown in table 2. Thus, hydrolysis under acidic and basic conditions follows a first-order reaction.

**HPTLC**

The order of degradation reaction was established by different methods, like substitution, graphical, and half-life methods, for acid hydrolysis (1 N) by determining recovery at different time intervals i.e. 2,4,8,16,32 h shown in table 2 and fig. 12, respectively.

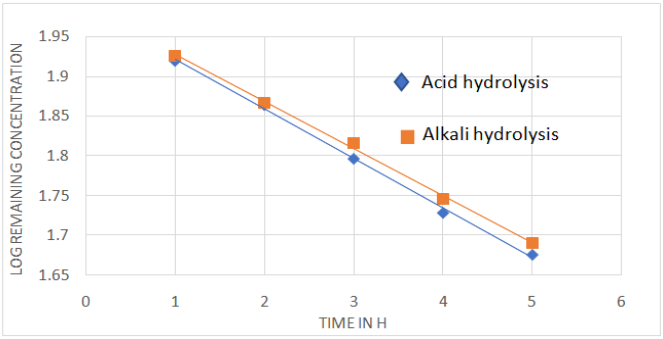



**Fig. 11: Kinetic curves for the hydrolytic degradation reaction**

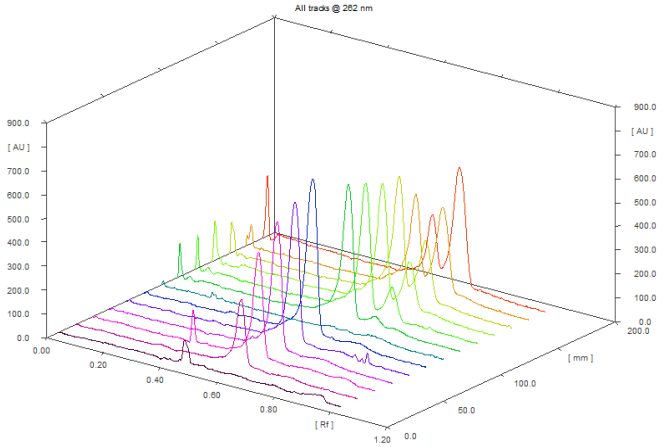



**Fig. 12: 3D Densitogram of the order of Acid hydrolysis (Track 1 blank, track 2-6 standard linearity, track 7 acid blank, track 8 at 0 min, track 9 at 2 h, track 10 at 4 h, track 11 at 8 h, track 12 at 16 h, track 13 at 32 h)**

**a) Substitution method**

For the substitution method, the following formula was used to determine the rate constant K,

$$K=\frac{2.303}{t}\log\frac{co}{ct}$$

Where, K= rate constant, t= time in h, co= initial concentration, ct= concentration at time t

Since the value of K is fairly constant for acid hydrolysis shown in table 2. Thus, acid follows a first-order reaction.

**b) Graphical method**

When the Log of remaining concentration was plotted against time, a straight line was obtained for acid hydrolysis. A straight line confirms a first-order reaction. Thus, acid follows a first-order reaction. The Kinetic curve for the hydrolytic degradation reaction is shown in fig. 13.

**c) Half-life method**

The half-life is calculated by the following formula,

$$t_{1/2}=\frac{0.693}{K}$$

Where, K= rate constant, $t_{1/2}$=half-life

The half-life is found to be independent to initial concentration for acid hydrolysis shown in table 2. Thus, acid follows a first-order reaction.

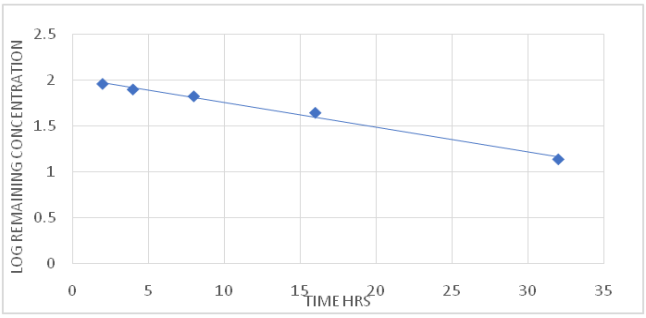



**Fig. 13: Kinetic curve for the hydrolytic degradation reaction**

**Table 2: Results of kinetic study data**

| System | Stress type | Time h | % remaining | Rate constant, K | Regression equation | $R^2$ | Half-life ($t_{1/2}$) |
|---|---|---|---|---|---|---|---|
| HPLC | Acid | 0 | 100 | - | y=-0.0624x+1.9845 | 0.9980 | - |
| | | 1 | 83.20 | 0.1830 | | | 3.78 |
| | | 2 | 73.21 | 0.1555 | | | 4.45 |
| | | 3 | 62.65 | 0.1558 | | | 4.44 |
| | | 4 | 53.55 | 0.1560 | | | 4.44 |
| | | 5 | 47.43 | 0.1491 | | | 4.64 |
| | Alkali | 0 | 100 | - | y=-0.0591x+1.9867 | 0.9981 | - |
| | | 1 | 84.38 | 0.1697 | | | 4.08 |
| | | 2 | 71.55 | 0.1670 | | | 4.14 |
| | | 3 | 65.5 | 0.1408 | | | 4.92 |
| | | 4 | 55.78 | 0.1458 | | | 4.75 |
| | | 5 | 49.04 | 0.1425 | | | 4.86 |
| HPTLC | Acid | 0 | 100 | - | y=-0.027x+2.0225 | 0.9913 | - |
| | | 2 | 89.79 | 0.052 | | | 13.32 |
| | | 4 | 78.12 | 0.061 | | | 11.36 |
| | | 8 | 65.82 | 0.052 | | | 13.32 |
| | | 16 | 43.59 | 0.051 | | | 13.58 |
| | | 32 | 13.64 | 0.062 | | | 11.17 |

**Linearity and range**

The linearity of the proposed method was evaluated according to the ICH guidelines. For HPLC Roxadustat showed linearity in the concentration range of 2.5–25 µg/ml. The linearity equation obtained was y=85569x+98059, where y is the peak area and x is a concentration of Roxadustat (µg/ml), and $R^2$= 0.9994, respectively. The residual plot of Roxadustat is shown in fig. 14. And for HPTLC, Roxadustat showed linearity in the concentration range of 500-2500 ng/band. The linearity equation obtained was y=5.0726x+5054.9, where y is the peak area and x is the amount spotted (ng/band), and $R^2$= 0.9957, respectively. The residual plot of Roxadustat is shown in fig. 15.

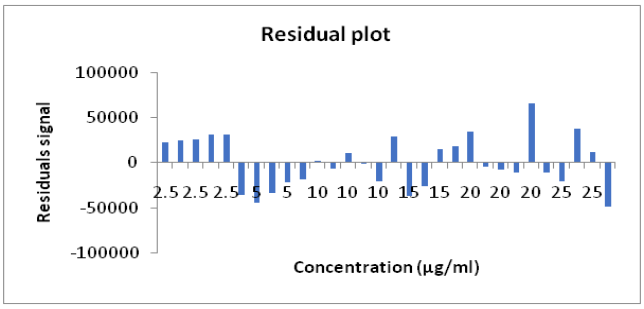



**Fig. 14: Residual plot of HPLC**

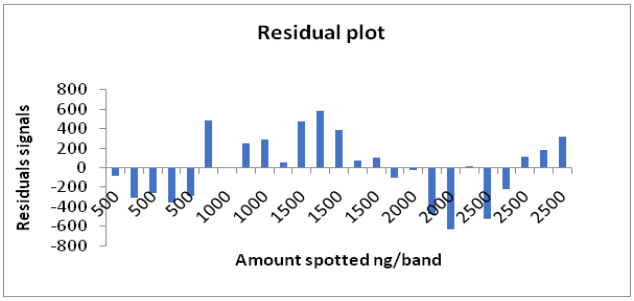



**Fig. 15: Residual plot of HPTLC**

The plot of residuals without any trend proves the linearity of the working range [20].

The plot of residuals without any trend proves the linearity of the working range.

**Assay**

**HPLC and HPTLC**

The assay was carried out using a spiked blend. Assay procedure repeated twice (n=2). For HPLC, the chromatogram was recorded and the % drug content was found to be 101.10±1.11 (% RSD) and for HPTLC the densitogram was recorded and the % drug content was found to be 100.41±0.57 (% RSD), respectively.

**Accuracy**

**HPLC**

The percent recovery for Roxadustat was found to be in the range as shown in table 3.

**HPTLC**

The percent recovery for Roxadustat was found to be in the range as shown in table 4 and the densitogram of accuracy studies is shown in fig. 16, respectively.

**Precision**

**HPLC and HPTLC**

Intraday and interday precision were performed. For HPLC % RSD was found to be 0.67% and 0.79% respectively. In HPTLC % RSD was found to be 1.35% and 1.44%, respectively.

**Limit of detection (LOD) and limit of quantitation (LOQ)**

**HPLC and HPTLC**

LOD and LOQ were calculated by the formula method. For HPLC the LOD and LOQ were found to be in the range i.e. 0.13 µg/ml and 0.39 µg/ml, respectively. And for HPTLC the LOD and LOQ were found to be in the range i.e.70.64 ng/band and 211.13 ng/band respectively.

**Table 3: Accuracy (% recovery) studies**

| S. No. | Amount from a spiked blend (µg/ml) | Amount of standard added (µg/ml) | Total amount of the drug (µg/ml) | Mean % recovery | % RSD |
|---|---|---|---|---|---|
| 1 | 10 | 8 | 18 | 100.20 | 0.79 |
| 2 | 10 | 10 | 20 | 101.83 | 0.20 |
| 3 | 10 | 12 | 22 | 99.50 | 0.40 |

**Table 4: Accuracy (% recovery) studies**

| S. No. | Amount from a spiked blend (ng/band) | Amount of standard added (ng/band) | Total amount of the drug (ng/band) | Mean % recovery | % RSD |
|---|---|---|---|---|---|
| 1 | 1000 | 800 | 1800 | 101.8 | 1.09 |
| 2 | 1000 | 1000 | 2000 | 101.52 | 0.72 |
| 3 | 1000 | 1200 | 2200 | 101.64 | 0.97 |

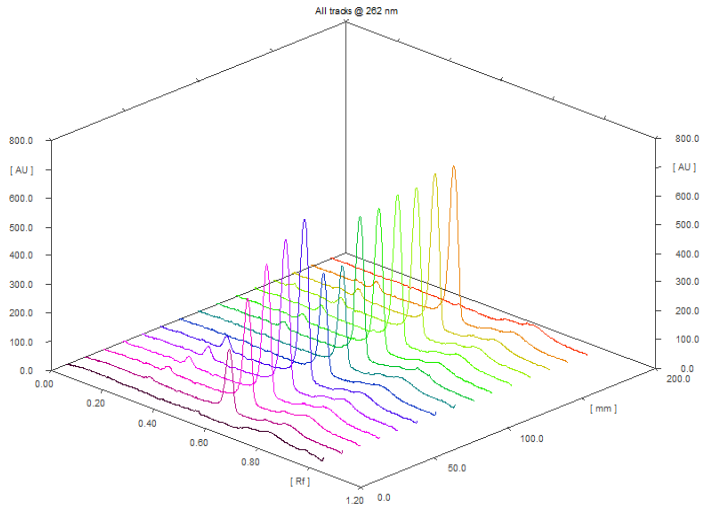



**Fig. 16: 3D Densitogram of accuracy (% recovery) (Track 1,15 methanol blank; track 2-6 standard linearity; track 7,8 assay 1000ng/band; track 9-10 standard addition @80%, track 11-12 std. addition @100%, track 13-14 std. addition @120% respectively)**

**Table 5: HPLC robustness studies**

| S. No. | Parameter | Conditions | % RSD |
|---|---|---|---|
| 1 | Mobile phase ratio | 68:32 | 1.69 |
| | Methanol: Phosphate buffer (70:30 *v/v*) (±2 ml) | 72:28 | 1.71 |
| 2 | Flow rate (1 ml/min) (±0.05 ml/min) | 0.95 | 1.52 |
| | | 1.05 | 1.82 |
| 3 | Phosphate Buffer (pH 5) (±0.2) | 4.8 | 1.41 |
| | | 5.2 | 1.63 |

**Table 6: HPTLC robustness studies**

| S. No. | Parameters | Conditions | %RSD |
|---|---|---|---|
| 1 | Mobile Phase ratio(±0.2 ml) | (4.8:5.2:0.5*v/v/v*) | 1.60 |
| | (Toluene: Ethyl acetate: Glacial acetic acid) | (5.2:4.8:0.5*v/v/v*) | 1.68 |
| 2 | Effect of time from spotting to development | Immediately after spotting | 1.70 |
| | | After2h | 1.52 |
| 3 | Effect of time from development to scanning | Immediately after development | 0.82 |
| | | After2h | 0.79 |
| 4 | Saturation time | 10 min | 1.75 |
| | (±5 min) | 20 min | 1.40 |
| 5 | Wavelength | 260 nm | 0.87 |
| | (±2 nm) | 264 nm | 1.02 |

**Robustness**

For HPLC and HPTLC in robustness, one factor at a time was changed. It was observed that the %RSD for peak area was found less than 2 %, which confirmed that the methods developed were robust. The results of the robustness study are shown in tables 5 and 6, respectively.

**Statistical analysis**

By applying paired t-test, a comparison of stress degradation by using HPLC and HPTLC methods has been done. As calculated t value (0.66) was found to be less than the table t value (2.45) [21]. We can conclude that the null hypothesis is accepted and there is no statistically significant difference in stress degradation monitored by HPLC and HPTLC methods.

## CONCLUSION

In the current work, simple, rapid RP-HPLC and HPTLC analytical methods for the determination of Roxadustat in the presence of its degradation products have been developed. Roxadustat was found to be sensitive to acid, alkali, and oxidative degradation conditions and relatively stable in neutral, thermal, UV, and fluro degradation conditions. The degradation product peaks were found in acid and alkali degradation. Kinetic studies show that acid hydrolysis and alkaline hydrolysis showed first-order kinetics. The developed methods were found to be precise, robust, accurate, sensitive, and reproducible for the stability study as per ICH Q2 (R1) guideline. The information presented herein could be very useful while developing formulation procedures to prevent hydrolytic degradation. It can be used as a routine quality control test.

## ACKNOWLEDGMENT

The authors are thankful to the principal and the management of the AISSMS College of Pharmacy, Pune, Maharashtra, India for providing the required facilities for research work.

## FUNDING

Nil

## AUTHORS CONTRIBUTIONS

MCD designed the work. JAS contributed for the analysis and data collection parts of the work. MCD and JAS contributed to the interpretation of the results.

## CONFLICT OF INTERESTS

Declared none

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