DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR THE ESTIMATION OF ELTROMBOPAG IN BULK AND TABLET FORMULATION

ABSTRACT:

Aims: A simple, gradient RP- HPLC method has been developed and validated for Eltrombopagin bulk and tablet formulation. **Methodology:** Successful estimation was carried out for the drug product was developed on $C_{(18)}$ column reversed-phase using acetonitrile: methanol: water in the ratio (55:35:10 %. v/v) as mobile phase composition. The flow rate was adjusted to 1ml /minute and the absorption maxima were observed at 230 nm utilizing Shimadzu SPD-20A Prominence UV-Vis detector. Eltrombopag drug showed a good and precise linearity in the range 2-10 µg/mL. **Results:** The HPLC, assay showed the purity ranging 99.07 to 100.17% for tablet formulation. The mean percentage purity was 99.76%. The chromatographic retention time of Eltrombopag was found to be 3.10 minutes. The statistical analysis showed the method accuracy. Various forced degradation studies were conducted on Eltrombopag to examine the stability of the drug. The developed method validated as per International Conference on Harmonization (ICH) guidelines. **Conclusion:** The developed method can be successfully applied for estimation and determination of Eltrombopag in bulk and tablet formulations.

Keywords: *Eltrombopag, RP-HPLC, UV, validation and forced degradation.*

1. INTRODUCTION

Eltrombopag is an orally bioavailable thrombopoietin receptor agonist that has been approved for the treatment of thrombocytopenia in patients with chronic immune thrombocyto penicpurpura (ITP). It is a medication that has been developed for the conditions leading to thrombocytopenia (abnormally low platelet counts). It is also being investigated for the treatment of thrombocytopenia due to other aetiologies including chronic liver disease and hepatitis C virus. [1-6]. The thorough literature survey revealed that a few normal and RP-HPLC methods for Eltrombopag in respective dosage forms are available, but all of these methods are specific to the bulk drugs. The degradation study was conducted to examine the proposed assay technique. Eltrombopag was put into different stress conditions to perform degradation studies. The drug solution was treated with acid, base, oxidative, dry and wet heat and direct sun light (photolytic stress) [7-11]. According to the proposed method the resulting drug samples were examined. The proposed aim of the study was to develop simple, accurate, specific and precise RP-HPLC method for the estimation of Eltrombopag in the bulk and pharmaceutical tablet formulation.

2. Materials and methods

2.1. Chemicals

The Eltrombopag reference standard (RS) was purchased from Sigma, Germany. The Revolade (Eltrombopag 50mg) tablet marketed drug of Glaxo Smith Kline Ltd, purchased from Preston, local Pharmacy, UK. The HPLC grade acetonitrile and ethanol was purchased from Sigma, German.

2.2. RP-HPLC INSTRUMENTATION

Shimadzu LC-20 AT HPLC system, using SPD-10 detector (SPD- M20A, Japan). A Zodiac $C_{(18)}$ column (50mm x 4.6mm, 5µm) with Pore size 95Å. The column temperature was maintained at 27ºC and the flow rate was 1ml/min. The injection volume was 20µl, 230nm was set as a wavelength and the HPLC run time was set for 10 minutes.

2.3. PREPARATION OF MOBILE PHASE

Approximately 350 ml of methanol was transferred into 1 liter volumaetric flask and 550 ml of acetonitrile and 100 ml of HPLC grade water was added and mixed thoroughly by shaking and the pH was adjusted to 6 by gradual adding of 0.5N phosphoric acid, the resulting solution was filtered with 0.45µ membrane filter. The final mobile phase was prepared by adding the ratio of (55:35:10 % v/v) acetonitrile: methanol: water.

2.4. PREPARATION OF ELTROMBOPAG STOCK SOLUTION

1mg Eltrombopag reference standard was weighed accurately and taken in 100 ml volumetric flask and mixed with 100 ml of mobile phase solution. The resulting solution was kept in the sonicator for 15 minutes. The concentration of 2-10 µg/mL was achived by diluting the standard stock solution with mobile phase. Eltrombopag powder were very freely soluble in water.

2.5. PREPARATION OF SAMPLE SOLUTION

10 tablets of marketed sample of Eltrombopag weighed accurately and powder equivalent of 50 mg transferred into 50ml volumetric flasks and dsolved with 50 ml of mobile phase and the resulting solution was filtered through Whatman 1 filter paper. Further dilutions were made based on the required concentrations.

2.6. SOLUTION STABILITY

The prepared drug solution stability was analysed during the time of analysis and also repeated the same analysis method on same day with different time intervels. The same analysis was repeated after 24hrs by keeping the drug solution under laboratory temperature (37 ± 1°C) and in refrigeration (5 ± 1°C).

2.7. FORCED DEGRADATION STUDY

Eltrombopag were put into different stress conditions to perform degradation studies. The degradation study were conducted to examine the proposed assay technique. Eltrombopag was freely soluble in methanol and also methonol is the portion of mobile phase, so methol was used as solvent. In all the experiments Eltrombopag contents equivalent to 5mg Eltrombopag was weighed. According to the stock solution procedure the solutions was prepared. 6 µg/ml of Eltrombopag was taken for every analysis. The drug solution was treated with acid, base, oxidative, dry and wet heat and direct sun light (photolytic stress). According to the proposed method the resulting drug samples were examined.

2.8. ACID DEGRADATION STUDY

The stability of Eltrombopag in acidic state were examined by treating with different concentrations of Hydrochloric acid 0.1N to 4N HCL. Solution of 6 µg/ml Eltrombopag was taken for this study was treated with 4N hydrochloric acid in presence of methanol. The treated drug solution was kept in dark chamber at 37°C for 12 hours.

2.9. ALKALI DEGRADATION STUDY

The stability of the Eltrombopag in alkaline condition were examined by treating with different concentrations of sodium hydroxide 0.1N to 4N NaOH. Solution of 6 µg/ml Eltrombopag were taken for this study was treated with 4N sodium hydroxide in presence of methanol. The treated drug solution was kept in dark chamber at 37°C for 12 hours.

2.10. OXIDATION STUDY

The stability of Eltrombopag under oxidative condition using hydrogen peroxide was examined. Solution of 6 µg/ml Eltrombopag was taken for this study was treated using 20 % H_2O_2 in presence of methanol. The treated drug solution was incubated at 37ºC for 12 hours.

2.11. WET HEAT STUDY

0.25mg of Eltrombopag taken for this study was treated with 2ml of HPLC grade water and the resulting solution was kept in dark chamber at 37°C for 12 hours. 6 µg/ml of Eltrombopag concentration were prepared as same as the preparation of stock solution procedure.

2.12. DRY HEAT STUDY

To conduct a dry heat analysis the drug sample was prepared by approximately 0.25gm of Eltrombopag powder in a clean aluminium foil and kept in an oven at 37°C for 12. The resulting Eltrombopag was weighed and solutions were prepared same as the preparation of stock solution procedure, 6 µg/ml of Eltrombopag was taken for the analysis.

2.13. PHOTO STABILITY STUDY (SUN LIGHT)

0.25gm of Eltrombopag was taken on a glass dish and exposed to direct sunlight. Exposing Eltrombopag over a period of 4 hours and carried the testing. The resulting Eltrombopag was weighed and solutions were prepared as same as the preparation of stock solution procedure, 6 µg/ml of Eltrombopag was taken for analysis.

3. RESULTS AND DISCUSSION

3.1 METHOD OPTIMIZATION

Chromatogram with good shape peaks and good retention time showed the good resolution for Eltrombopag and forced degradation products. The proposed method was to identify the number of degradation products formed during the stressed conditions. The typical RP-HPLC conditions are presented in Table 1. The good separation of Eltrombopag and the products degraded peak under stressed conditions shows the success of the method. The HPLC chromatogram of Eltrombopag standard and Eltrombopag is presented in figure 1 and 2.

Table 1. HPLC conditions for estimation of Eltrombopag.

Parameters	Description
Column	Zodiac $C_{(18)}$ column (50mm x 4.6mm, 5µm)
Column temperature	27 ± 1°C
Mobile phase	Acetonitrile: Methanol: Water (55:35:10 % v/v)

Detection	Photodiode array detection at 230 nm
Injection volume	20 µl
Flow rate	1 ml min^{-1}

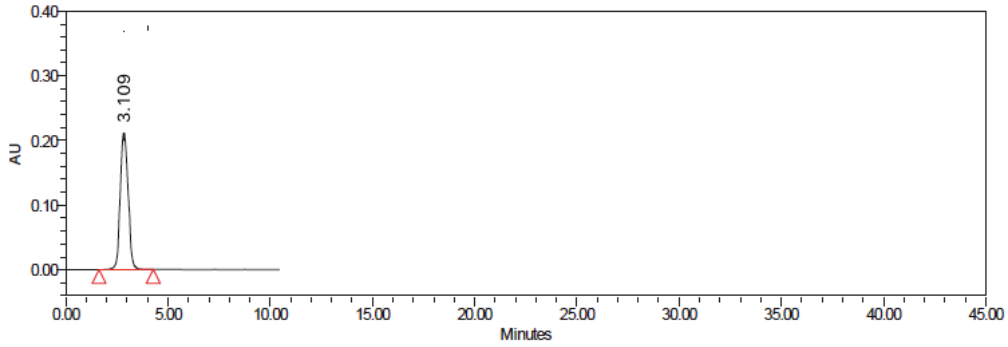

Fig.1. A Typical Chromatogram of Eltrombopag Standard

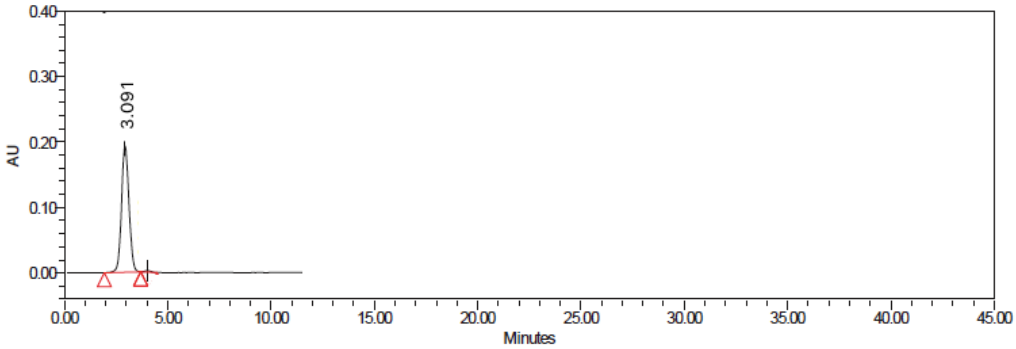

Fig.2. A Typical Chromatogram of Eltrombopag tablet formulation

3.2. METHOD VALIDATION

The method proceeded to achieve sensitive, easy and economical for determination and estimation by HPLC from tablet formulation. Based on the ICH recommended guidelines and the experimental was validated.

3.2.1 LINEARITY

The proposed method linearity was examined for five different concentrations. The concentration ranges from 2-10 µg/ml. The Eltrombopag standard linearity was determined by the plotting graph concentration vs peak area. By peak area as a functional of analyte concentration linearity was evaluated for Eltrombopag. The linearity graphs presented in figure 3, and data presented in Table 2. The system suitability is demonstrated by the linearity analysis.

Table 2. HPLC linearity data for Eltrombopag

Concentration (µg/ml)	Peak area
2	1544.47
4	3027.55
6	4523.50
8	6097.45
10	7649.79

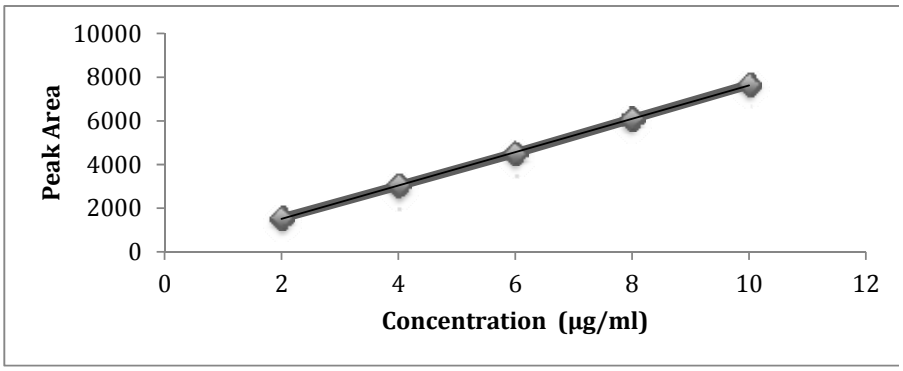

Fig. 3. Calibration graph of Eltrombopag 2-10 µg/ml precision

3.2.2 ACCURACY

The recovery experiment showed the accuracy of the method. The good recovery showed the method was accurate. The analysis for recovery was performed by known amount of Eltrombopag working standard added to pre-analyzed solution of the formulation in the test concentration range of (40%, 60% and 100 %). For each recovery level three samples was prepared and repeated for 3 consecutive days. The statistical results for recovery study are well within the range (S.D. < 2.0). The Eltrombopag tablet formulation recoveries results are presented in Table 3.

Table 3. Recovery studies of Eltrombopag tablet formulation.

S No	Drug	Amount of Drug present in preanalyzed Sample (µg/ml)	Amount of Standard drug (RS) added (µg/ml)	Amount of drug recovered (µg/ml)	% Recovery	Mean recovery in Percentage
1.	Eltrombopag	6	4 6 10	9.73 11.97 15.72	99.27 99.75 99.28	99.43

3.2.3 PRECISION

The proposed method precision (repeatability) experiment results of are shown in Table 4. In the proposed method intraday and interday precision was examined by analyzing the responeses of the sample on the same day for 4 repeatations and 3 alternate days for 2-10 µg/ml concentration range of Eltrombopag. The obtained results are represented in % RSD. The % CV of the proposed method was precise as the values < 1.0 % for the repeatability study. The precision data are presented in Table 5.

Table 4. Method precision data of Eltrombopag by RP-HPLC method

Eltrombopag 6µg/ml (n=4)	Retention time	Area
1	3.101	4523.22
2	3.091	4493.20
3	3.107	4634.79
4	3.097	4553.52
Mean	3.10	4551.18
S.D [a]	0.0211	102.71
% CV[b]	0.68	2.27

n=4 observations

Table 5. Intermediate precision data of Eltrombopag by RP-HPLC method

Eltrombopag µg/ml	Inter-day measured mean area ± S.D.[a]	%CV[b] (n[c]=4)	Intra-day measured mean area ± S.D.[a]	%CV[b] (n[c]=4)
6	4557.25± 2.19	0.0872	4697.44± 4.65	0.0125
8	6019.75±2.22	0.0617	6172.75±2.15	0.0157
10	7613.12±2.11	0.0722	7576.12±4.26	0.1056

n[c] = 4 observations

3.2.4 SPECIFICITY

Specificity of chromatographic method was established by the separation of drug peak from the adjacent resolving peaks. Specificity was verified by running a blank, placebo and drug working solution to check any interference at the retention time of the standard peak. Specificity was further confirmed by inducing degradation of eltrombopag to acidic, basic, sunlight and temperature. The peak purity for each degradation product was established, which has shown efficient separation of drug (eltrombopag) peak from its degradation peaks. The Eltrombopag standard reference and the drug formulation shows the specificity of the method. The RP-HPLC chromatogram of Eltrombopag both bulk and the tablet formulation are presented in figure 1, 2. The Eltrombopag standard reference and tablet formaulation retention time was found to be 3.10 min. For the tablet formulation there was no excipient interference was detected, which shows the specificity of the method. The proposed method showed the ability to determine the analyte in presence of excipients.

3.2.5 LIMIT OF DETECTION AND QUANTITATION

The limit of detection and quantification for Eltrombopag is presented in table 6. Limit of detection (LOD) and limit of quantification (LOQ): LOD and LOQ were estimated by injecting the diluted known concentration solution, which gave minimum detectable peak area. This was multiplied thrice to get

LOD and by 10 times to get LOQ with suitable precession as per the International Conference on Harmonization guidelines. LOD and LOQ were calculated which was found at concentrations of 0. 75µg/mL and 1.15 µg/mL respectively.

Table 6. Results of Limit of detection & limit of quantification

Parameters	Eltrombopag
LOD (µg/ml)	0.75
LOQ (µg/ml)	1.15

3.2.6 THE SYSTEM SUITABILITY

For the system suitability parameters five repeats of standards and two repeats of sample preparation are injected, the data is presented in table 7. The Assay data of Eltrombopag presented in table 8. The obtained assay results are subjected to the coefficient of variation, statistical analysis, regression equation and standard deviation are presented in table 9.

Table 7. Results of system suitability parameters

SNo	Parameters	Eltrombopag
1.	Theoretical plates	5791
2.	Tailing factor	0.975
3.	Resolution factor	2.14
4.	Retention time	3.1± 0.2
5.	Calibration range or Linear dynamic range	2-10µg/ml

Table 8. Quantitative estimation (Assay) data of Eltrombopag

SNo	Drug	Label claim (µg/ml)	Amount found (µg/ml)	Mean amount found (mg/ ml)	Percentage purity (% w/w)	Mean purity (% w/w)	% Deviation
1.	Eltrombopag	50	50.11 49.67 50.01 49.93 50.14	49.97	100.17 99.40 100.11 99.07 100.04	99.76	+ 0.2 +0.1 +0.2 +1.0 +0.4

n= 4 observations

Table 9. Results of statistical parameters Statistical parameters

SNo	Parameters	Eltrombopag
1.	Standard deviation (SD)	1.47

2.	Relative standard deviation (RSD)	0.0566
3.	% RSD	0.676
4.	Standard error (SE)	0.02077
5.	Correlation Coefficient (r)	0.9993
6.	Slope (a)	24.510
7.	Intercept (b)	45.77
8.	Regression equationY = (aX+b)	Y = 24.510 X +45.77

4. FORCE DEGRADATION OF ELTROMBOPAGIN FORMULATION

Eltrombopag showed a high degradation in dry heat and wet heat study. Secondly moderate degradation was found in the photo stability (Sun light) condition. There was no degradation effect on oxidative stress. Table 10 indicates the degradation of Eltrombopag under acidic, alkali, wet heat, dry heat oxidative and photo stabily stress conditions. According to the ICH guidelines of the forced degradation study of Eltrombopag was examined.

Table 10. Summary of Force degradation of Eltrombopag by RP-HPLC method

SNo	Stress condition/ state	Time	% Assay ± S.D. [a](n[b]=5)
1	Acidic 4N HCL(37 °C)/ solution	12 hrs	99.012 ± 0.692
2	Alkali 4N NaOH (37 °C)/ solution	12 hrs	99.013± 0.774
3	Wet heat (37 °C)/ solution	12 hrs	92.167±1.013
4	Dry heat (37 °C)/ solid	12 hrs	83.941±1.643
5	Oxidative 20 % H_2O_2 (37 °C)/ solution	12 hrs	99.607±1.122
6	Photo stability/ solid	4 hrs	88.668±1.013

S.D.[a] is standard deviation for n[b] = 5 observations

4.1. ALKALI DEGRADATION

The stability of the Eltrombopag in alkaline condition was examined. There was no degraded product was separated from Eltrombopag. The chromatogram of alkali-degraded result was compared with the formulation and standard chromatogram. The result shows around 1–2 % of the Eltrombopag drug was degraded and in alkaline condition the drug was highly stable.

4.2. ACID DEGRADATION

The stability of the Eltrombopag in acid condition was examined. The chromatogram of acidic condition product was compared with the formulation and standard chromatogram. The result shows around 1–2 % of the Eltrombopag drug was degraded. The result shows that drug in acidic condition were highly stable.

4.3. OXIDATION

The stability of the Eltrombopag in oxidation condition was examined. It was observed that no degradation was taken place on exposure to 20 % H_2O_2 for 12 hrs. The chromatogram of oxidation product was compared with the formulation, standard chromatogram and blank H_2O_2. No degradation product peaks were observed. The 20 % H_2O_2 peak time observed at RT 3.105 and in the peak height and area has a significant decrease with time, presented in figure 4. The result shows that the drug were highly stable to oxidative conditions.

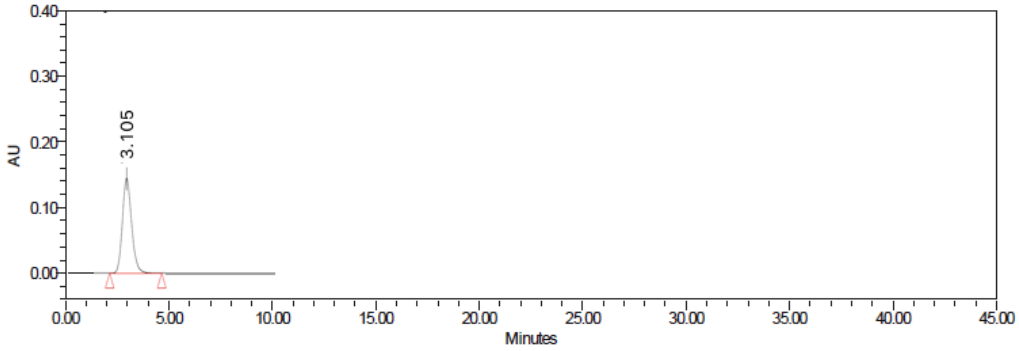

Fig. 4. Chromatogram of Eltrombopag under oxidation condition by RP-HPLC method

4.4. WET HEAT (HYDROLYSIS)

The stability of the Eltrombopag in neutral condition, 7–8 % drug degradation was observed after 12 hrs of incubation at 37ºC. The chromatogram of wet heat degraded product was compared with the formulation and standard chromatogram presented in figure 5. In the wet degraded chromatogram Eltrombopag peak area and height has a significant decreased and two different degradation peak were observed at 7.4 and 14.6 mins.

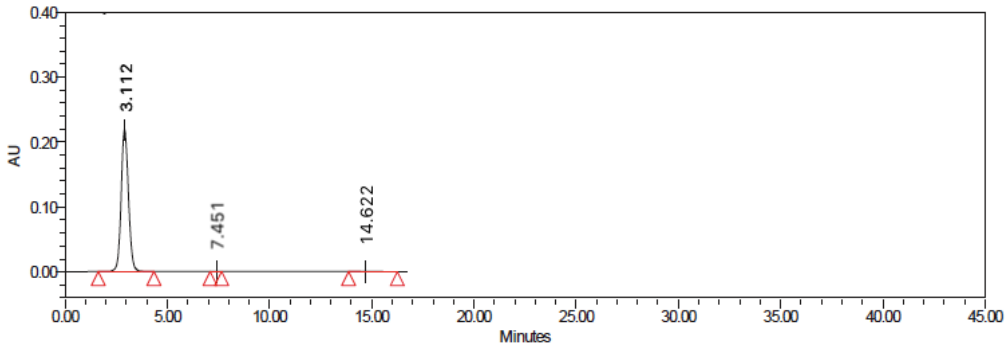

Fig.5. Chromatogram of Eltrombopag under wet heat condition by RP-HPLC method

4.5. DRY HEAT

The chromatogram for Eltrombopag in dry heat shows the drug was slightly unstable as compare to acid, base and wet degradation. Around 15-17 % drug degradation was observed. The chromatogram

of dry heat degraded product was compared with the formulation and standard chromatogram. In the dry degraded chromatogram the drug decomposed into minor degradation products. The result shows major significant decreases in the peak height and peak area with time, three different degradation peaks were observed between 5-14 mins, presented in figure 6.

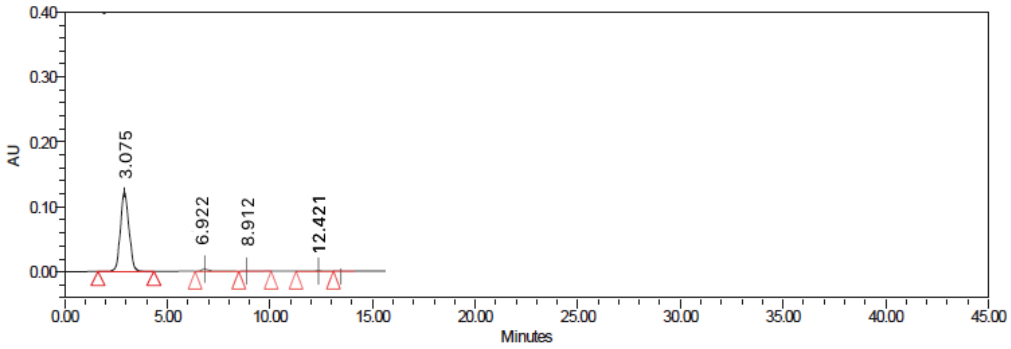

Fig. 6. Chromatogram of Eltrombopag under dry heat condition by RP-HPLC.

4.6. PHOTO STABILITY (SUN LIGHT)

The chromatogram for Eltrombopag under photo stablilty study was found to be unstable after drug exposure to direct sunlight for 4 hours. Almost 10-12% of the drug degraded in 4 hours. One minor drug degradation peak was observed at 4.5 minute and also there was a significant decrease in the drug peak height and peak area were observed which is presented in figure 7.

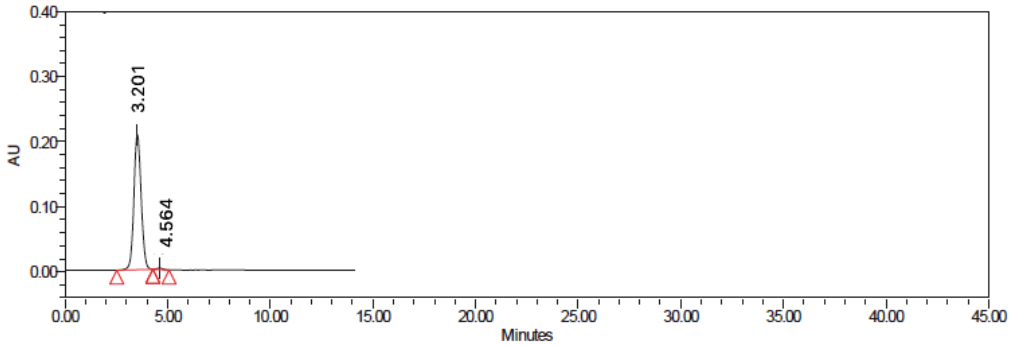

Fig.7. Chromatogram of Eltrombopag under Photo stability condition by RP-HPLC

4. CONCLUSION

The force degradation study was performed according to the guidelines of International Conference on Harmonization (ICH), The developed RP-HPLC method shows the accuracy, sensitive and stability of the method. The developed method is rapid, reproducible. The developed method can be used for the routine analysis for Eltrombopag tablet formulations.

REFERENCES

1. Deng Y, Madatian A, Wire M.B, Bowen C, Park J.W, Williams D, Peng B, Schubert E, Gorycki F, Levy M, Gorycki P.D. Metabolism and disposition of eltrombopag, an oral, non peptide thrombopoietin receptor agonist, in healthy human subjects, Drug Metab Dispos. 2011;39(9):1734–1746.

2. Serebruany V.L, Eisert C, Sabaeva E and Makarov L. Eltrombopag (Promacta), a thrombopoietin receptor agonist for the treatment of thrombocytopenia: current and future considerations, Am. J. Ther. 2010; 17(1):68–74.

3. Allred A.J, Bowen C.J, Park J.W, Peng B, Williams D.D, Wire M.B and Lee E. Eltrombopag increases plasma rosuvastatin exposure in healthy volunteers, Br. J. Clin. Pharmacol. 2011; 72(2) :321-329.

4. Wire M.B, Mclean H.B, Pendry C, Theodore D, Park J.W and Peng B. Assessment of the pharmacokinetic interaction between eltrombopag andlopinavir–ritonavir in healthy adult subjects. Antimicrob. Agents Chemother. 2012; 56(6):2846–2851.

5. Williams D.D, Peng B, Bailey C.K, Wire M.B, Deng Y, Park J.W, Collins D.A, Kapsi S.G and Jenkins J.M. Effects of food and antacids on the pharmacokinetics of eltrombopag in healthy adult subjects: two single-dose, open-label, randomized-sequence, crossover studies. Clin. Ther. 2009; 31(4):764–776.

6. Rambabu M, Ramakrishna G, Nageswara R.P, Sridhar S, Srinubabu M and Ajitha M. Liquid chromatography–tandem mass spectrometric assay for eltrombopag in 50 µL of human plasma: A pharmacokinetic study. J. of Pharm Biomed Anal. 2014;98:68–73.

7. Mahaboob B.D, Venkata R.G, Shobha R.T, Susithra E and Rajasekhar C. RP-UPLC-MS assay method for eltrombopag: Application in pharmaceuticals, human plasma and urine samples, Asian J. Chem. 2015; 27(12):4615–4619.

8. Monika B and Saranjit S. Development of validated stability indicating assay methods-critical review. J. of Pharm Biomed Anal. 2002; 28:1011-1040.

9. ICH harmonized tripartite guideline, stability testing of new drug substances and products, Q1A (R2), 2003.

10. ICH of technical requirements for the registration of pharmaceutical for the human use, validation of analytical procedures: text and methodology, ICH, Q2 (R1), 2005.