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RP-UPLC-MS Assay Method for Eltrombopag: Application in Pharmaceuticals, Human Plasma and Urine Samples

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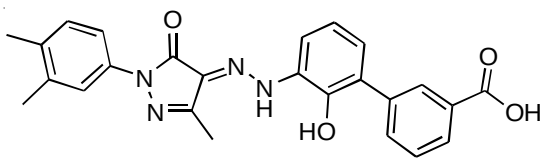
A simple, accurate, rapid and precise method was developed using UPLC coupled to mass detector (UPLC-MS) for stability and estimation of eltrombopag (EMP) in its bulk and dosage forms, human plasma and urine samples. Instrumentation used for this method was highly sophisticated and of superior technology in terms of speed, separation, solvent usage and analysis cost. The efficient separation of drug from its degradation products was achieved by using Acquity UPLC BEH column C_{18} (50 × 2.1 mm, 1.7 µm) with linear gradient elution of mobile phase (0.1 % formic acid in water & acetonitrile) 97:03 % v/v at initial gradient and flow rate of 0.6 mL/min. The chromatogram was analyzed at a wavelength of 230 nm (λ_{max}) using PDA detector and further confirmed by mass (*m/z*) using waters SQD (Single Quadrapole Detector-3100 mass range 100 amu-1000 amu) with a run time of 4.5 min. The linearity range was performed from 20 to 100 µg/mL (R^2 = 0.999) with regression of y = 433.88x + 35.4. The variation of precision study was found to be < 1 %. Eltrombopag was showing more sensitivity towards the acidic degradation. The formed degradative products were identified by mass spectrometry (UPLC-MS) and the mode of degradation was reported. The dosage form and bulk drug samples were subjected to forced degradation and analyzed as per ICH Q1A (R2) and Q2 (R1) guidelines.

Keywords: Eltrombopag, UPLC, Mass spectrometry, Forced degradation, ICH.

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[1-3]. Eltrombopag should be used only in patients with immune thrombocyto penicpurpura whose degree of thrombocytopenia and clinical condition increases the risk for bleeding[4-6].

As per the previous investigations, eltrombopag was used in quantitative estimation in human plasma at different concentrations and its pharmacokinetic properties were determined by using different analytical techniques. But the developed methods are not so simple, accurate and rapid. A thorough literature review illustrates the availability of few analytical methods for determination of the drug eltrombopag in biological samples, including liquid chromatography/mass spectrometric[7-13] and high performance liquid chromatographic (HPLC) methods. However, quantification of eltrombopag in pure form (bulk drug) and its pharmaceutical dosage form is a newer approach and has not been reported so far. Hence, we aimed at exploring a rapid method evidenced with stability indicating study and attempted its application to human plasma and urine samples using UPLC-MS as per International Conference on Harmonization (ICH) guidelines[14,15]. It was found, that using this rapid method more number of quality control samples could be analyzed in human plasma and urine (Bio-ARD) samples within less time and with more quality and precision. The proposed method was found to be useful for structural identification in drug discovery department using mass detector.



Chemical structure of eltrombopag

EXPERIMENTAL

The chromatographic experiment was carried out using an UPLC™ system (ACQUITY) consisting a binary pump with high pressure gradient having low delay volumes. UPLC binary solvent manager (BSM) and injector could produce pressure up to 15000 psi, whereas in HPLC, the pressure ranges from 4500 to 6000 psi. UPLC decreases the analysis duration 10 times over the HPLC with low solvent consumption and is connected with a high sensitive PDA detector (very narrow flow cell path length). The use of hybrid technology (BEH-Bridged Ethylene Hybrid) column with sub 2 micron particle size (C_{18}, 50 × 2.1 mm, 1.7 µm) offers significant advantages over present HPLC instruments. It is coupled with Waters Mass SQD-3100 (Single Quadrupole Detector) having ESI source wherein the results are obtained using the software Mass Lynx V 4.1. By using mass detector, the mass spectrum (*m/z*) of drug and the impurities arising from forced degradation at both modes (ESI +ve and –ve modes), could be obtained which helps in identifying the nature of the molecule at various conditions. Aliquot samples of 1 µL were injected into the column, kept an ambient temperature[16-20].

Eltrombopag reference standard was obtained from Dr. Reddy's Laboratories Limited (Hyderabad, India). A strip of seven tablets of eltrombopag olamine (Brand name: PrRevolade® label claim 25 mg per tablet, manufactured by Glaxo Operations UK Ltd., Ware, UK. Batch no: REV0201) was purchased from reputed pharmacy in Hyderabad. HPLC grade acetonitrile, methanol and analytical-mass grade formic acid (fluka) were purchased from Sigma-Aldrich Ltd. (Bangalore, India). Highly purified Milli-Q Water used for the UPLC/MS from Milli-Q water purification system procured from Millipore (Bangalore, India).

Preparation of solutions

Standard & sample solution preparations: The standard solution of eltrombopag was prepared by dissolving 25 mg of accurately weighed sample in 25 mL volumetric flask by using a mixture of methanol:water (9:1, diluent). The calibration samples were prepared from working standard solution to obtain the final concentrations of 20, 40, 60, 80, 100 µg/mL.

The sample solution was prepared by weighing seven tablets. The weight of each tablet was found to be approximately 370 mg (PrRevolade®). This was crushed to a fine powder; powder equivalent to the drug (25 mg) was taken in 25 mL volumetric flask and dissolved with the methanol:water. The solution was sonicated for 30-40 min to achieve complete dissolution. It was then filtered using 0.45 µm membrane filter and the filtrate was used for performing validation parameters by diluting with diluent.

Extraction of drug from plasma and urine: The drug was extracted from human plasma by protein precipitation (PPT) method and from urine samples by LLE technique. Blank plasma was collected from healthy volunteer's blood, centrifuged at 7000 rpm for 10 min and the collected plasma was stored at -20 °C.

Extraction of plasma samples by protein precipitation method: After thawing the plasma to room temperature, the drug was spiked (2 % to the plasma volume) and from that, 0.5 mL of spiked plasma was mixed with 1 mL of 0.1 % formic acid in acetonitrile taken in a centrifuge tube. It was vortexed vigorously for 10 min and again centrifuged for the same time at 7000 rpm from which 1 µL supernant was used for the following method.

Extraction of urine samples by LLE method: Blank urine was collected from healthy volunteers and the drug was spiked. To 0.5 mL of spiked urine sample, 3 mL of ethyl acetate: *n*-hexane (85:15 v/v) was added and vortexed for 10 min followed by centrifugation for 10 min at 7000 rpm. About 2.5 mL of the supernatant was taken from the prepared extracted solution, evaporated under nitrogen gas at 40 °C and reconstituted with 0.5 mL of diluent. 1 µL of this extracted sample was then loaded into the system in a similar manner.

Chromatographic method: The analysis of present study was carried out with acquity BEH column (C_{18}, 50 × 2.1 mm, 1.7 µm) using the mobile phase (0.1 % formic acid in water as aqueous buffer and 0.1 % formic acid in acetonitrile as organic solvent) which was sonicated for 10 min and filtered through 0.45 µm membrane filter. A linear gradient program at a flow rate of 0.6 mL/min, scanning wavelength of 230 nm and run time of 4.5 min was used. Methanol:water (9:1) was used as a diluent with 1 µL sample injection volume. Before running the instrument, the column was equilibrated for 15 min with initial gradient flow.

RESULTS AND DISCUSSION

Earlier investigations were performed with chromatographic conditions that are cost effective and time consuming with less productivity with higher flow rates and more run time and required pH adjustment of mobile phase. In comparison, the developed assay method was simple, rapid and cost effective in terms of mobile phase consumption, preparation and run time. Initial studies were done with acidic, neutral and basic buffers using different dimension of columns. It was found that in the trial of neutral buffer (5 Mm ammonium acetae:acetonitrile) with C_{18}, bad peak shape and poor ionization in mass spectrometry were observed, in the basic buffer trial (5 Mm ammonium bicarbonate:acetonitrile) with C_{18}, good peak shape but extra neighboring peaks were observed with low sensitivity. Finally, high quality chromatographic results were observed using BEH-C_{18} column (50 × 2.1 mm, 1.7 µm) with 0.1 % formic acid in water and acetonitrile. Using this developed assay method, the drug eltrombopag was well separated from its degradative products generated from forced degradation.

System suitability: System suitability was performed by running five injections of standard solution (100 µg/mL) repeatedly and the system was verified. Every day freshly prepared working solutions from stock solution were used for UPLC injections. The system performance was checked by acquiring the results and characteristic parameters such as tailing factor, theoretical plates, retention time and relative standard deviation which were found to be within their permissible limits (Table-1).

Accuracy: Accuracy expresses the closeness of individual measured value to the nominal value. The % recovery of developed test method was performed for drug sample (eltrombopag

TABLE-1
RESULTS OF SYSTEM SUITABILITY

Eltrombopag system suitability		
% RSD Standard area	0.21	NMT 2 %
Theoretical plates	32000	NLT 2000
USP tailing	1.07	NMT 2
% RSD RT	0.00	NMT 2 %

tablets) as well as for commercial tablet by the method of standard addition at three concentration levels (50, 100, 150 µg/mL) in order to calculate the recovery percentages in triplicates (Tables 2 and 3).

TABLE-2
ACCURACY-RECOVERY STUDY OF ELTROMBOPAG

Sample No.	Spiked conc. (µg)	Measured conc. (µg)*	Recovery (%)*	RSD (%)*
1	50 (50 %)	49.882	99.76	
2	100 (100 %)	99.709	99.70	0.1
3	150 (150 %)	149.850	99.90	

*Each value is a mean of three readings

TABLE-3
ANALYSIS OF ELTROMBOPAG
COMMERCIAL FORMULATION (TABLETS)

Sample No.	Formulation	Labeled claim (mg)	*Amount found (mg)	*Recovery (%)
1	PrRevolade®	25	24.76	99.04

*Each value is a mean of three readings

Precision: Precision of the present study was expressed at three levels: intermediate precision, repeatability and reproducibility. Intermediate precision was evaluated on different days, with different instrument, different analyst and repeatability was done in the same day. The results of the precision study are shown in Table-4 below. The % RSD of the total precision study was found to be 0.18-0.91.

TABLE-4
RESULTS OF PRECISION

Type of precision		Mean peak area ± SD	RSD (%)	Conc. (µg/mL)
Analyst	Analyst-1	23001 ± 209	0.91	
	Analyst-2	23178 ± 170	0.73	
Instrument	Instrument-1	22593 ± 40	0.18	
	Instrument-2	22763 ± 65	0.29	100
Inter day		21176 ± 196	0.93	
Intra day		21666 ± 96	0.45	
Method		22733 ± 119	0.52	

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, peroxide, UV chamber, sunlight and temperature. The peak purity for each degradation product was established using PDA detector which has shown efficient separation of drug (eltrombopag) peak from its degradation peaks.

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 Q2 (R1)[14,15]. LOD and LOQ were calculated which was found at concentrations of 1.43 µg/mL and 4.30 µg/mL respectively.

Linearity and range: Linearity test solutions of 20, 40, 60, 80, 100 µg/mL were prepared from a stock solution using diluent, injected into the UPLC-MS system keeping the injection volume of 1 µL constant. The calculated graph from Fig. 1 was linear over the 20-100 µg/mL concentration range (Table-5) and the obtained regression equation was found to be at slope intercept of y = 433.88x + 35.4 with correlation coefficient of 0.999.

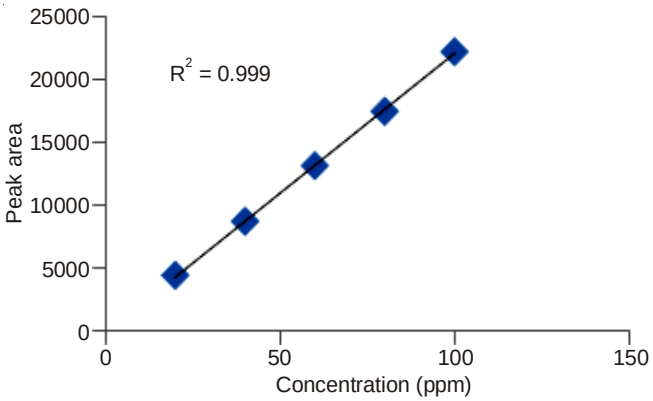


Fig. 1. Calibration curve

TABLE-5
RESULTS OF LINEARITY

Concentration (ppm)	Area
20	4365
40	8669
60	13109
80	17445
100	21671

Robustness: Robustness of the present study was performed by changing the flow rate (± 0.1) and mobile phase concentration (± 0.01). A small difference in peak areas, retention time were observed for flow rate changes (% RSD 0.61-1.41), insignificant difference for mobile phase concentration (% RSD 0.36-0.77) and there was no impact on the % assay of the chromatographic method.

Forced degradation studies: Forced degradation has been carried out to confirm that during stability study or throughout the shelf life, any degradation product if found will not interfere in the main peak and with known impurities. In addition, forced degradation study will help to identify the type of degradation pathways (through acidic, alkali, oxidative, photolytic and dry heat) for each of degradants[14]. For all degradation studies, the solutions were prepared at initial concentrations of 1 mg/mL, after degradation, it was neutralized and diluted to give final concentration of 50 µg/mL using diluent (methanol:water) as shown in Table-6.

TABLE-6
RESULTS OF FORCED DEGRADATION

Stress conditions	Degradation (%)	Positive mode (*m/z*)	Negative mode (*m/z*)
Treated with 5N HCl solution for 5 h on a water bath at 40 °C	51.61	457	455
Treated with 5N NaOH solution for 5 h on a water bath at 40 °C	2.25	235, 229	233,213
Treated with 10 % H_2O_2 solution 5 h on a water bath at 40 °C	1.40	295.7	-
Treated with heat at oven about 50 °C for 3 h	0.00		
Exposed to sunlight for 24 h	1.40	459,545	-
Exposed to UV light for 24 h (≥ 200 w h/m^2)	0.00		

In acid degradation, the drug (eltrombopag) was decomposed to nearly 52 % with *m/z* ratio of 457 (+ve mode) and 455 (-ve mode). It was identified that the drug (eltrombopag) undergoes esterification. In basic degradation, the degradation *m/z* ratios of 229, 213 were obtained which might be due to fragments of the eltrombopag as illustrated in **Scheme-I**.

Analysis of drug in human plasma and urine samples: The tablet dosage form (eltrombopag) availability was checked in human plasma and urine samples by this chromatographic method. Plasma was collected from blood of healthy human bodies (age between 25-30 years, non-alcoholic, non-smokers and not taking other medicines). Blood samples of healthy volunteers were collected in evacuated glass tubes; plasma was separated by centrifuging the blood samples at 7000 rpm for 10 min and stored at -20 °C. After thawing the plasma to room temperature, the drug (eltrombopag) was spiked, extracted by protein precipitation and assayed at the concentration levels. In a similar manner, urine samples were collected from healthy volunteers, spiked with drug (eltrombopag) dosage form of desired concentration, extracted by LLE technique and analyzed by this chromatographic method. The linearity and recovery study was also done. The assay results of plasma and urine samples are summarized in Tables 7 and 8. Based on the obtained results, this method was also applied for Bio-Analytical study in Bio-AR&D Department.

TABLE-7
ANALYSIS OF ELTROMBOPAG IN PLASMA SAMPLES BY THE PROPOSED METHOD

Eltrombopag	Amount spiked (mg)	Amount found (mg)	Recovery (%)
Bulk drug	1	0.8523	85.20
Dosage form	1	0.9138	91.40

TABLE-8
ANALYSIS OF ELTROMBOPAG IN URINE SAMPLES BY THE PROPOSED METHOD

Eltrombopag	Amount spiked (mg)	Amount found (mg)	Recovery (%)
Bulk drug	1	0.8857	88.60
Dosage form	1	0.9526	95.30

Conclusion

The reported method emphasizes the need for a preliminary treatment and substantiates UPLC-MS method as a simple, sensitive, accurate, precise and reliable validated method for the estimation of eltrombopag in bulk drug and also in tablet formulation with minimal run time of 4.5 min. By this method, the drug was well separated from all degradation products which were well identified by mass spectrometry. Hence, UPLC-MS method could find an application as a convenient technique for the ongoing process control analysis, human plasma and urine sample analysis, without any interference of the additives/excipients generally present in the pharmaceutical formulations. Moreover, the defined method is compatible for Bio-AR&D department LC-MS/MS analysis with less run time and cost effective in terms of its low solvent consumption with more productivity.

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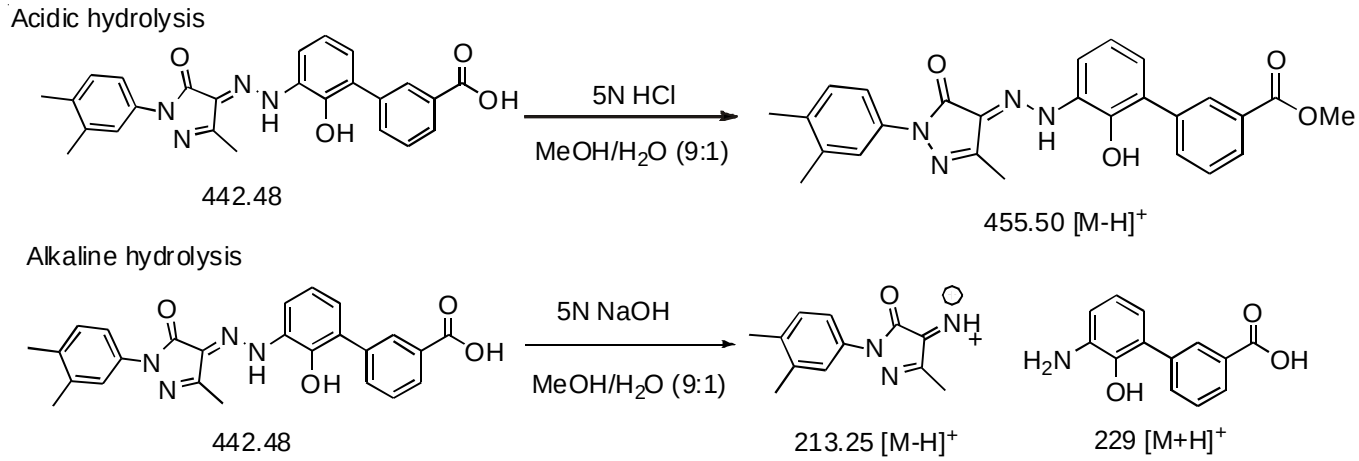

Scheme-I

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