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Original Article

SIMULTANEOUS ESTIMATION OF EPALRESTAT AND METHYLCOBALAMININ BULK AND TABLET DOSAGE FORM BY USING RP-UPLC

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ABSTRACT

To Study and develop an accurate, precise and linear Reverse Phase Ultra Performance Liquid Chromatographic (RP-UPLC) method for simultaneous quantitative estimation of Epalrestat and Methylcobalaminin Bulk and tablets werevalidate as per ICH guidelines. The optimized method uses a reverse phase column, Dikma column (1.7 x 2.1mm, 5□m), a mobile phase of0.05Mpotassium dihydrogen orthophosphate buffer (pH 3.0) and Methanol in proportion 30:70 %v/v, flow rate of 0.3 ml/min and a detection wavelength of 220 nm using a UV detector. The developed method resulted in Epalrestat eluting at 0.895 min and Methyl Cobalaminat 1.317 min. Epalrestat exhibited linearity in the range 150-750 µg/ml, while Methylcobalaminexhibited linearity in the range 1.5-7.5 µg/ml. The precision is exemplified by relative standard deviations of 1.4% for Epalrestat and 0.9% for Methylcobalamin. Percentage Mean recoveries were found to be in the range of 95-105 by percentage method during accuracy studies.

Keywords: *Epalrestat, Methylcobalamin, RP-UPLC, Method Development, Validation.*

INTRODUCTION

Epalrestat (Figure 1), chemically 2-[(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2 sulfanylidene-1,3-thiazolidin-3-yl]acetic acid an aldose reductase inhibitorused for the treatment of diabetic neuropathy [1,2]. It reduces the accumulation ofintracellular sorbitol which is believed to be the cause of diabetic neuropathy [3].

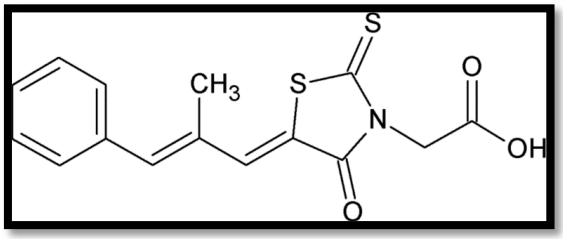

Figure 1: Structure of Epalrestat

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Methyl cobalamin (Figure 2), chemically carbanide;cobalt (2+);[(2R,3S,4R,5S)-5-(5,6-dimethyl benzimidazol-1-yl)-4-hydro-xy-2-(hydroxymethyl)oxolan-3-yl]-1-[3-[(1R,2R,3R,5Z,7S,10Z, 12S,13S,15Z,17S,18S,19R)-2,13,18-tris(2-amino-2-oxoethyl)-7, 12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19-octa meth-yl-2,7,12,17-tetrahydro-1H-corrin-24-id-3-yl]propanoyl amino]p ropan-2-yl hydrogen phosphate is a cobalamin, a form of vitamin B12. It differs from cyanocobalamin in that the cyanogroup at the cobalt is replaced with a methyl group [4]. Methyl cobalaminfeatures an octahedral cobalt (III) centre and can be obtained as bright red crystals [5].

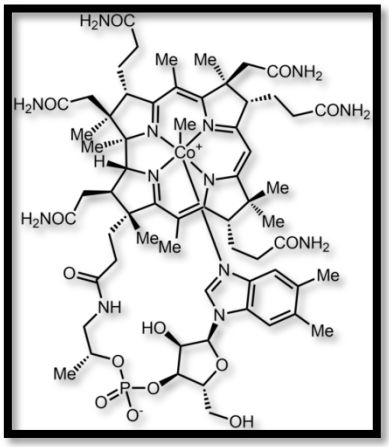


Figure 2: Structure of Methylcobalamin

In literature review there are a few analytical methods were reported for estimation of Epalrestat and Methylcobalamin alone or in combination with other drugs in pharmaceutical dosage forms. No other methodwas available for the simultaneous estimation of Eparlestat and Methylcobalamin by using RP-HPLC or RP-UPLC, but only one method was available in this combination by using UV-Spectrophotometry [6]. Eparlestat and Methylcobalamin in individual or by combined with other drugs by using UV and RP-HPLC[7-18]. The main objective of the present work describes a simple, rapid, precise and accurate reversed phase stability indicating UPLC method for the simultaneous determination of Epalrestat and Methylcobalamin in combined pharmaceutical dosage forms as per ICH guidelines [19, 20].

MATERIALS AND METHODS

Generic product of fixed dose combination of Epalrestat450 mg and Methylcobalamin4.5 mg (Epalrica-M Tablet) was purchased from the local market. Reference standards (API's)Eparlestat and Methylcobalamin were received as gift samples from Hetero Labs. Methanoland potassium dihydrogen orthophosphate was purchased from Fisher Scientific, Mumbai.

Instrumentation:

The UPLC system (Waters) comprised of quaternary pump, auto sampler, PDA detector with 2695 separation module, controlled by EMPOWER 2 Software,Dikma column (1.7 x 2.1mm, 5m) was used for analysis.An electronic balance (Afcoset ER-200A), pH meter (Adwa – AD 1020),hot air oven, ultra sonicator and millipore Vacuum Filtration Assembly were also used in the study.

Preparation of solutions:

Preparation of 0.05M Phosphate Buffer: Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

Mobile Phase Optimization: Accurately measured 300 ml of above buffer and 700 ml of Methanol HPLC (30:70% v/v) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Wave length selection: UV spectrum of 10µg/ml Epalrestat and Methylcobalamin in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 220nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Dikma column (1.7 x 2.1mm, 5m) was found to be ideal as it gave good peak shape and resolution at 0.3 ml/min flow.

Preparation of the Epalrestat & Methylcobalamin Standard & Sample Solution:

Standard Stock Solution Preparation: Accurately weigh and transfer 1500 mg of Epalrestat and 15 mg of Methylcobalamin working standard into a 100 ml clean dry volumetric flask &added about 70 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.3 ml of Epalrestat (450 µg/ml) and Methylcobalamin (4.5 µg/ml) of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Stock Solution Preparation: Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 1500 mg ofEpalrestat and 15mg Methylcobalamin (marketed formulation) sample into a 100mL clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.3 ml of Epalrestat (450 µg/ml) and Methylcobalamin (4.5 µg/ml)of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Method Validation:

The method validation was done by following the ICH guidelines. The followingvalidationcharacteristic parameters are accuracy, precision, linearity, specificity, robustness and degradation study.

Linearity and Range: Linearity of the method was studied by the injecting the mixed standard solutions with theconcentration ranges from 150-750µg/mlfor Epalrestatand 1.5- 7.5µg/ml for Methylcobalaminlevels of target concentrations wereprepared and injected six times into the UPLC system keeping the constant injection volume. The peak areaswere plotted against the concentrations to obtain the linearity graphs.

Precision: The precision of the optimized method was evaluated by carrying out six independent assays of testsample. %RSD of six assay values was calculated. Intermediateprecision was carried out the samples by usinganother instrument and with different analyst.

Accuracy: Accuracy was carried out by applying the method to drug sample (Epalrestat and Methylcobalamin combination of tablets) to which known amounts of Epalrestat and Methylcobalamin standard powder corresponding to 50, 100 and 150% of label claim was added, mixed and the powder was extracted and determined by the system in optimized mobile

phase. The experiment was performed in triplicate and percentage recovery, % RSD was calculated.

Limit of Detection and Quantification: The LOD and LOQ procedures were performed on samples contain very lower concentrations of analytes under the ICH guidelines. By applying the visual evaluation method, LOD was expressed by establishing the lowest concentration at which the analyte can be detected. LOQ was considered as the lowest concentration of analytes that can be detected and quantified, with acceptable accuracy and precision.

Robustness: Robustness was studied by evaluating the effect of small variations in the chromatographic conditions.

The conditions studied were flow rate altered by ±0.1ml/min, mobile phase composition with methanol ±5ml.

These chromatographic variations are evaluated for resolution between Epalrestat and Methylcobalamin.

Analysis of marketed formulation: The marketed formulation was assayed by above description. The peak areas were monitored at 220nm, and determination of sample concentrations were using by multilevel calibration developed on the same UPLC system under the same conditions using linear regression analyzed for Epalrestat and Methylcobalamin in the same way as described above.

System suitability: The system suitability parameters with respect of tailing factor, theoretical plates, repeatability andresolution between Epalrestatand Methylcobalaminpeaks were defined.

Specificity: The specificity of the analytical method is the ability of the method to estimate the analyte response inthe presence of additional components such as impurities, degradation products and matrix [21]. The peak purity of Epalrestatand Methylcobalamin were assessed by comparing the Retention time of standard Epalrestat and Methyl cobalamin good correlation was obtained between the Retention time of standard and sample of Epalrestat and Methylcobalamin.

The specificity method was also evaluated to ensure that there were no interference products resulting from forced degradation studies.

Forced degradation study:

Forced degradation or Stress testing of a drug substance will help to identify the degradation products, which can help to establish the intrinsic stability of the molecule.

All stress decomposition studies were performed at an initial drug concentration 450µg/mL of Epalrestatand4.5µg/mL of Methylcobalamin.

The Stability indicating study of Epalrestat and Methylcobalamin were undergoes acid, alkali and oxidation degradation,photolysis and heat condition.

Placebo Interference: The placebo (in the present of excipients in tablet) sample were prepared as per the testmethod and analyzed in the UPLC. It expressed there is no additional peaks at the retention time of Epalrestat and Methylcobalamin in the chromatograph it indicates that there is no placebo interference.

Acid Degradation: Sample was treated with 3ml of 0.1N hydrochloric acid and kept for 10hrs. After 10hrs thesolution was neutralized with 3ml of 0.1N sodium hydroxide, made the volume upto the mark with diluent and analyzed using UPLC.

Alkali Degradation: Sample was treated with 3ml of 1N sodium hydroxide and kept for 10hr. After 10hr thesolution was neutralized with 3ml of 1N hydrochloric acid, made the volume upto the mark with diluent and analyzed using UPLC.

Oxidative Degradation: Epalrestat and Methylcobalamin solutions of 30 and 75µg/ml were mixed with 3mL of 30%v/v aqueous hydrogen peroxide solution and kept for 10hrs. After 10hrs made the volume upto the mark with diluent and analyzed using UPLC.

Photolytic Degradation: The samples were kept under UV light for different time intervals (15mins – 7days)and made the volume upto the mark with diluent and analyzed using UPLC.

Thermal Degradation: Samples were heated at 800 C for 15mins - 60mins and 2200 C for 2-5mins andanalyzed.

RESULTS AND DISCUSSION

Method Development and Optimization:

The optimized chromatographic conditions are a combination of 0.05M potassium dihydrogen orthophosphate buffer (pH 3.0) and Methanol in proportion 30:70 %v/v at a flow rate of 0.3ml/min over Dikma column(1.7 x 2.1mm, 5µm). System suitability parameters such as Retention time(Rt), Theoretical plates(N), Peak area(A)and Resolution, the statistical data of parameters were calculated using Empower 2.0 software andthe results showed the performance parameters of the developed analytical method comply with USP requirements of system suitability(Table 1& Figure 3-5).

Table 1: Optimized chromatographic conditions

Instrument used	Waters UPLC with auto sampler and PAD or detector
Temperature	Ambient
Column	Dikma column (1.7 x 2.1mm, 5µm)
Buffer	0.05MPotassium dihydrogen ortho phosphate
pH	3.0
Mobile phase	Buffer : Methanol (30:70)
Flow rate	0.3 ml/min
Wavelength	220 nm

Injection volume	4 µl
Run time	10min
Retention Time	0.895 & 1.317

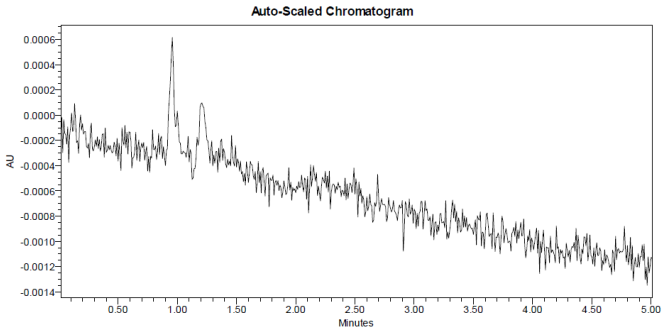


Figure 3: Blank Chromatogram

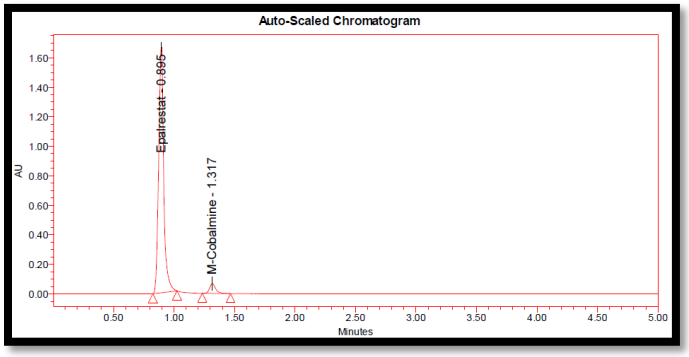


Figure 4: Standard chromatogram of Epalrestat and Methylcobalamin

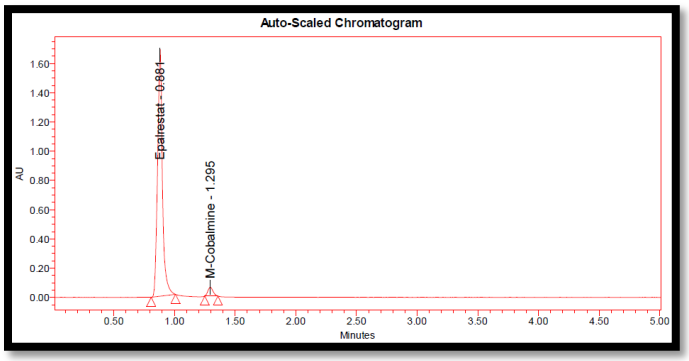


Figure 5: Sample chromatogram of Epalrestat and Methylcobalamin

Method Validation:

Precision: Repeatability and intermediate precision were checked by analyzing replicate composite reference solutions (n = 6) of known concentrations. The overall % RSD for peak response on day 1 and day 2 was checked against acceptable limits of ± 2% for repeatability and intermediate precision (Table 2).

Table 2: Precision results of Epalrestat and Methylcobalamin

Inj. No.	RT (min)	Peak Area	USP Plate Count	USP Tailing	RT (min)	Peak Area	USP Plate Count	USP Tailing
	Epalrestat				Methylcobalamin			
1	0.866	4669547	2315.99	1.14	1.277	205555	4074.58	1.23
2	0.870	4633682	2458.86	1.18	1.281	213714	3716.10	1.28
3	0.873	4711857	2422.02	1.14	1.285	202403	3867.05	1.14
4	0.873	4586290	2539.47	1.13	1.286	191233	3962.12	1.11
5	0.874	4690109	2433.68	1.17	1.287	197507	4061.01	1.22
6	0.876	4674377	2633.46	1.16	1.290	205189	4309.18	1.29
Mean		4660977.0				192600.0		
Std. Dev.		44751.8				13583.0		
% RSD		1.0				1.1		

The % RSD of peak areas (A) and retention time(Rt) for both analytes was less than 2.0.Resolution between two analytes was more than 3.0, tailing factor was less than 2.0 and the number of theoretical plates was more than 2000. The method was found to be suitable for simultaneous determination of Epalrestatand Methylcobalaminafter successful application of their estimation in tablet dosage form.

Accuracy: The accuracy of method was established by analysing the solutions of 3 concentrations (n=3) at 3 different levels (50%, 100%, 150%) of target assay concentration. The % recovery and relative standard deviation (RSD) were evaluated against acceptable limits of ± 2% (Table 3).

Table 3: Accuracy resultsof Epalrestat and Methylcobalamin

Drug Name	Recovery levels (%)	Conc. (µg/mL)	Area	% Recovery
Epalrestat	50		2403115	100.15
	100		4830189	100.65
	150		7218887	100.28
	Assay		4812173	100.36
Methylcobalamin	50		101992	100.87
	100		203933	100.84
	150		304854	100.50
	Assay		202009	100.73

Linearity and Range: For linearity assessment six solutions (n=3) with known concentrations of Epalrestat (150, 300, 450, 600 & 750 µg/ml) and Methylcobalamin (1.5, 3, 4.5, 6 & 7.5 µg/ml) were analyzed. Calibration curve was plotted between peak response on y-axis & concentration on x-axis. The relation between concentration and response was evaluated by least square linear regression method y = mx + c, where m is slope, y= peak area, c = intercept and x = concentration (Figure 6 &7, Table 4).

Table 4: Linearity data for Epalrestat and Methylcobalamin

S. No.	Epalrestat	Methylcobalamin		
	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area
1	150	1737625	1.5	93475
2	300	3100790	3	159362
3	450	4487985	4.5	220699
4	600	5851773	6	287360

5	750	7102614	7.5	351109
Slope		8987.x		42884x
Intercept		41186		29421
Correlation Coefficient (R2)		0.999		0.999

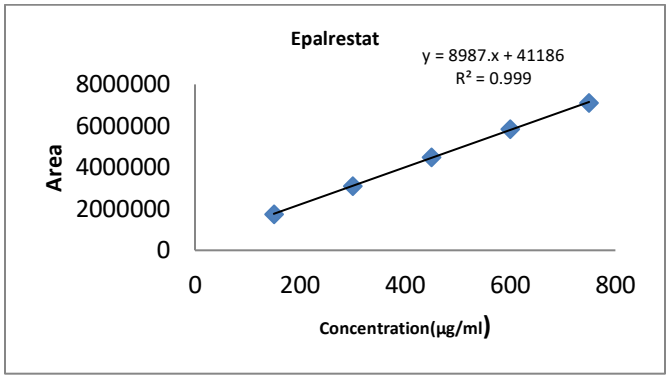


Figure 6: Linearity Curve of Standard Epalrestat

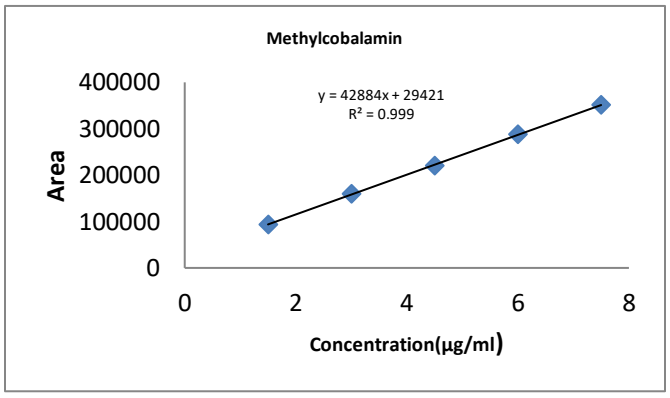


Figure 7: Linearity Curve of Standard Methylcobalamin

Robustness: Robustness of method was assured by analyzing replicates (n=6) of solution used for precision with small changes in chromatographic conditions such as composition of mobile phase, flow rate etc. The influence of variables is determined by evaluating % RSD against acceptable limit of ± 2% for peak response and Rt of each analyte (Table 5).

Table 5: Robustness study results ofEpalrestat and Methylcobalamin

Parameters	Adjusted to	Epalrestat		M-Cobalmine	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
Flow Rate (ml/min)	0.27	2101.88	1.13	4005.09	1.15
	0.3	2516.02	1.16	3904.77	1.25
	0.33	2796.68	1.09	3430.48	1.21
Mobile Phase Composition (Organic)	10% less	2543.39	1.13	3265.96	1.15
	*Actual	2516.02	1.16	3904.77	1.25
	10% more	2864.38	1.09	3542.38	1.21

* Results for actual flow (0.3ml/min) have been considered from Assay standard.

* Results for actual Mobile phase composition (30:70 Methanol: Buffer (ph-3) has been consideredfrom Accuracy stand.

Forced Degradation studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Epalrestat and Methylcobalamin using the proposed method.

Table 6: Percentage of degradation of Epalrestat and Methylcobalamin

Parameters	Epalrestat		Methylcobalamin	
	Area	% Degraded	Area	% Degraded
Standard	4789358		201829.1	
Acid	4674578	2.40	189789	5.97
Base	4528841	5.44	184262	8.70
Peroxide	4551776	4.96	185975	7.86
Thermal	4430886	7.48	180657	10.49
Photo	4557032	4.85	183737	8.96

Hydrolytic degradation under acidic condition:Pipette 0.3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials. The drug content was found to be degrading up to 2.4& 5.97% of Epalrestat and Methylcobalamin respectively in Acidic condition (Figure 7 & 8, Table 6).

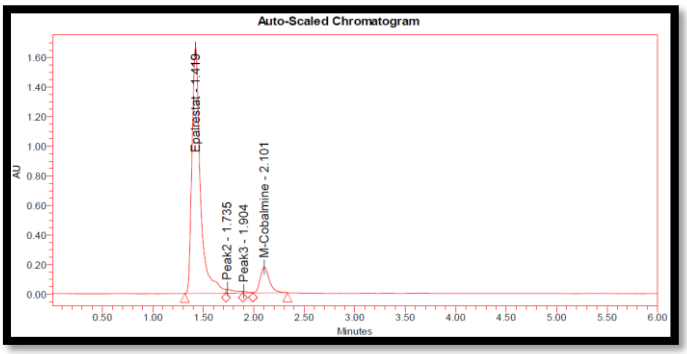


Figure 7: Chromatogram of Acidic Forced Degradation of Epalrestat and Methylcobalamin

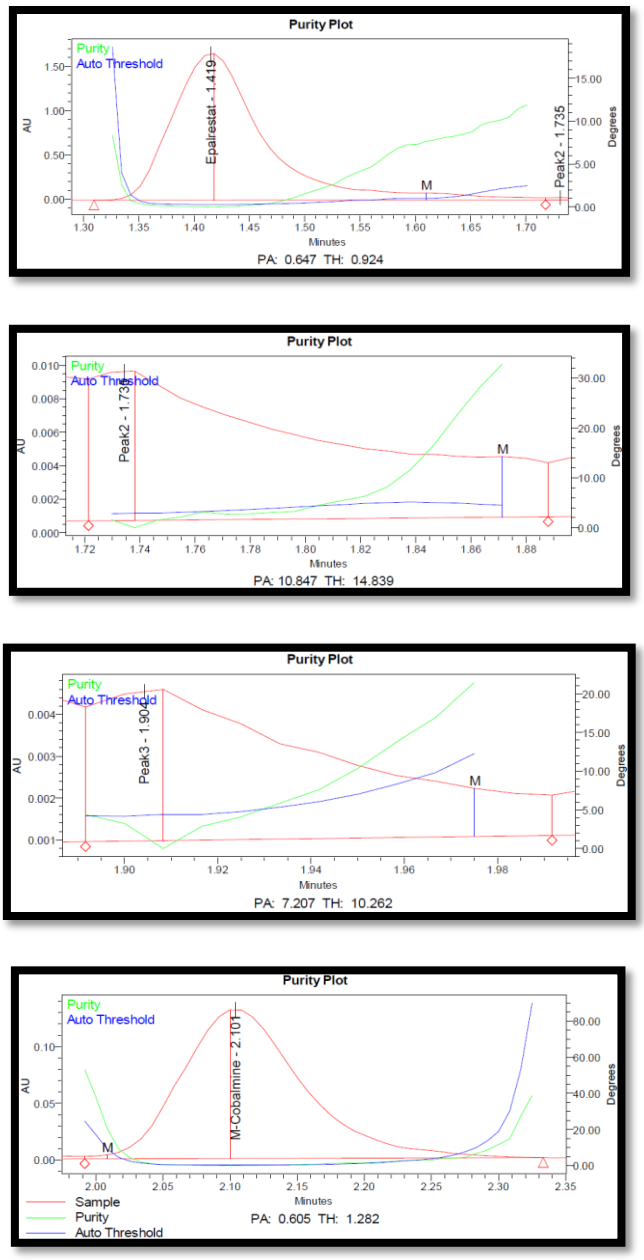


Figure 8: Purity Plots for Epalrestat and Methylcobalamin in Acidic Forced Degradation

Hydrolytic degradation under alkaline condition:Pipette 0.3 ml of above solution into a 10ml volumetric flask into a 10ml volumetric flask and add 3 ml of 0.1N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials. The drug content was found to be degrading up to 5.44 & 8.7% of Epalrestat and Methylcobalamin respectively in Alkali condition (Figure 9 & 10, Table 6).

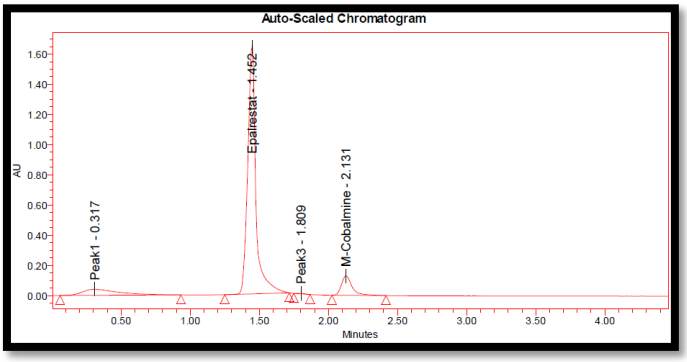


Figure 9: Chromatogram of Alkali Forced Degradation of Epalrestat and Methylcobalamin

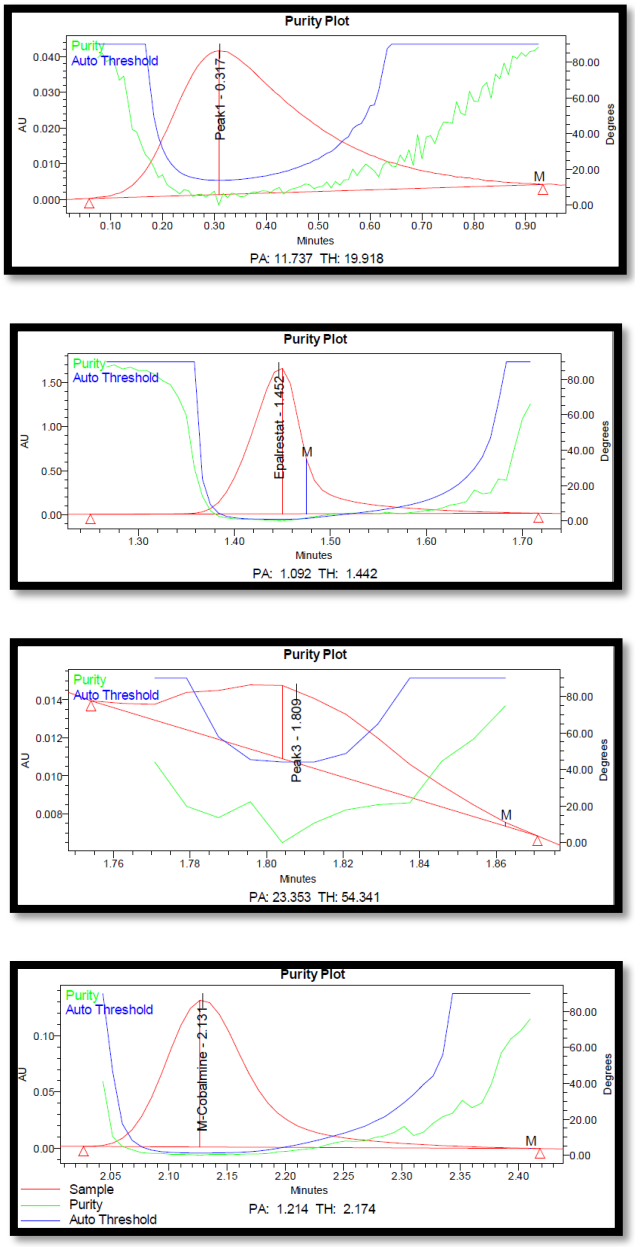


Figure 10: Purity Plots for Epalrestat and Methylcobalamin in Alkali Forced Degradation

Oxidative degradation: Pipette 0.3ml above stock solution 2 into a 10ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials. The drug content was found to be degrading up to 4.96& 7.86% of Epalrestat and Methylcobalamin respectively in Oxidative condition (Figure 11 & 12, Table 6).

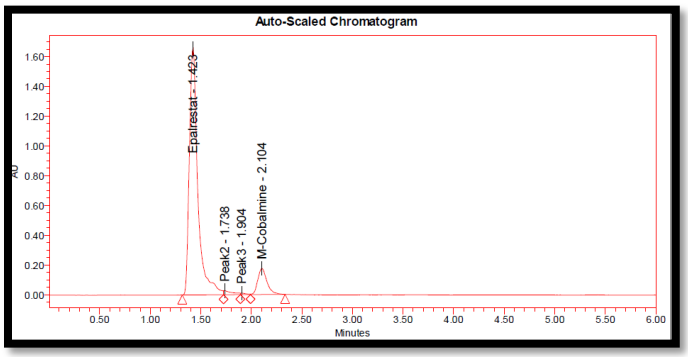
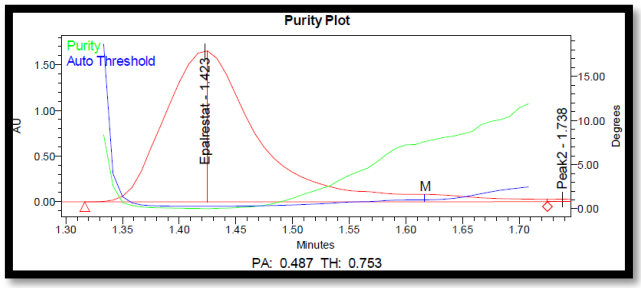
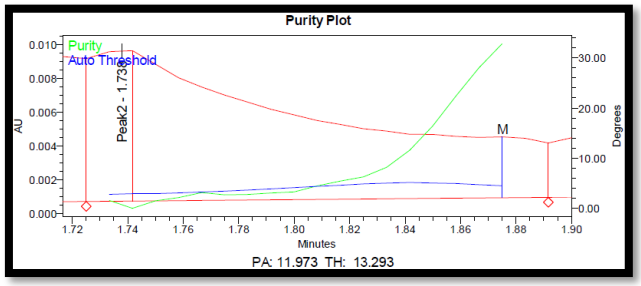
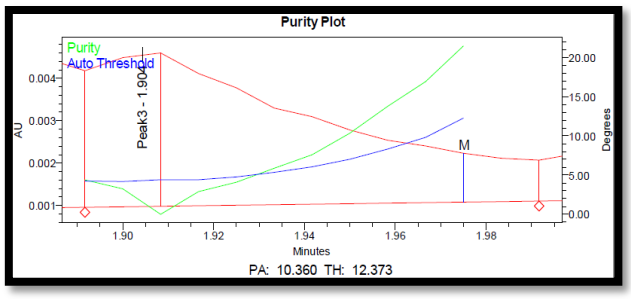


Figure 11: Chromatogram of Oxidative Forced Degradation ofEpalrestat and Methylcobalamin







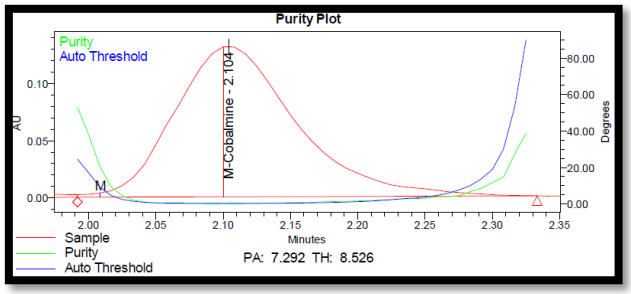


Figure 12: Purity Plots for Epalrestat and Methylcobalamin in Oxidative Forced Degradation

Thermal induced degradation: Epalrestat and Methylcobalam in sample was taken in petri dish and kept in Hot air oven at 1100 C for 24 hours. Then the sample was taken and diluted with diluents and injected into UPLC and analysed. The drug content was found to be degrading up to 7.48 &10.49% of Epalrestat and Methylcobalamin respectively in Thermal condition (Figure 13 & 14, Table 6).

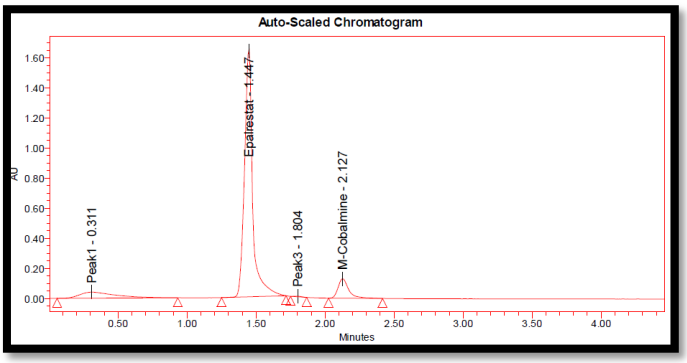
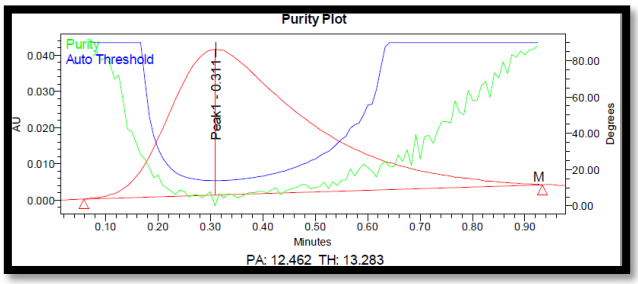


Figure 13: Chromatogram of Thermal Forced Degradation of Epalrestat and Methylcobalamin



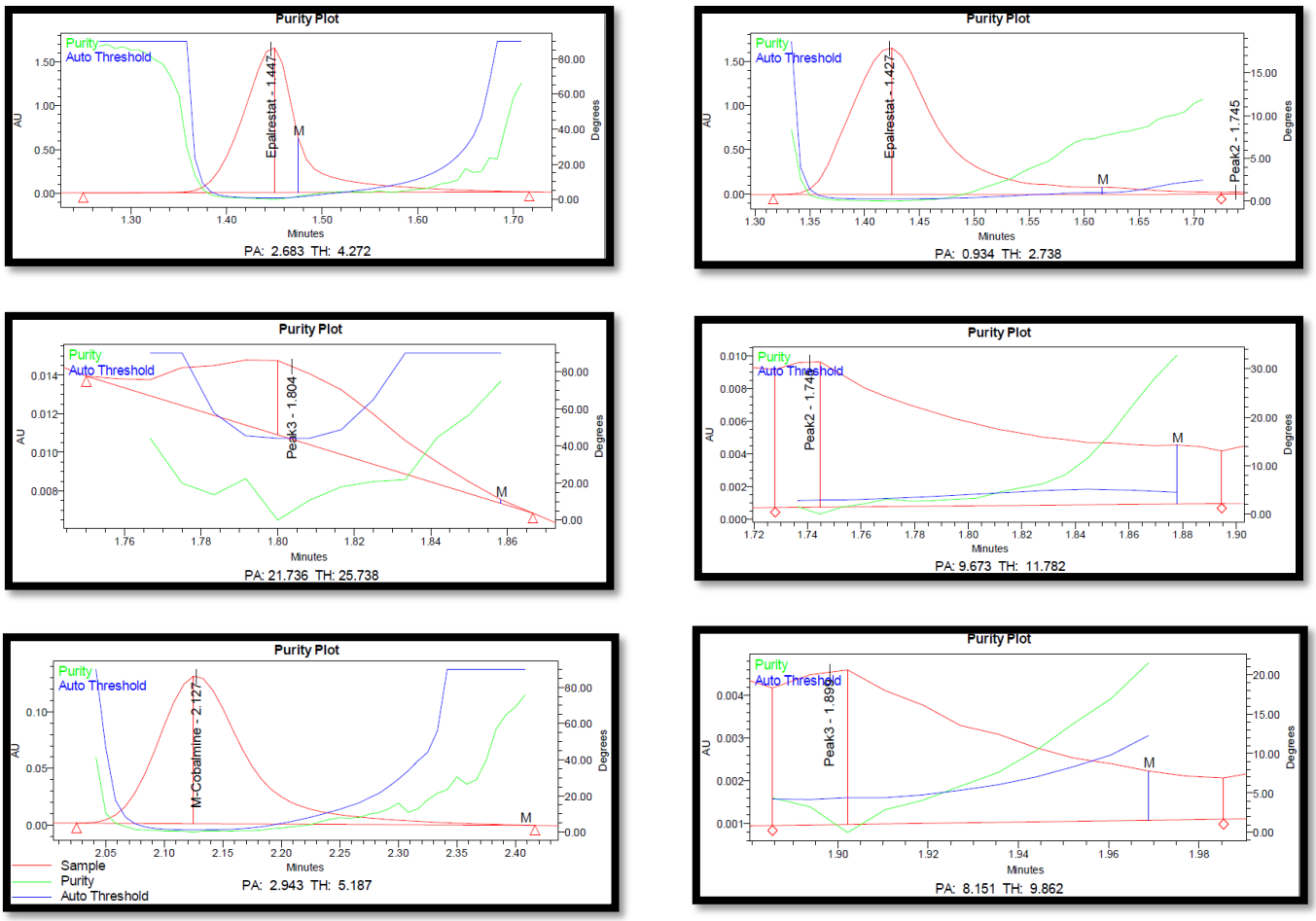


Figure 14: Purity Plots for Epalrestat and Methylcobalamin in Thermal Forced Degradation

Photolytic degradation study: Photolytic degradation study was performed by exposing the drug content in UV light for 15mins to 7days. The drug content was found to be degrading up to 4.85&8.96% of Epalrestat and Methylcobalamin respectively in Photolytic condition (Figure 15 & 16, Table 6).

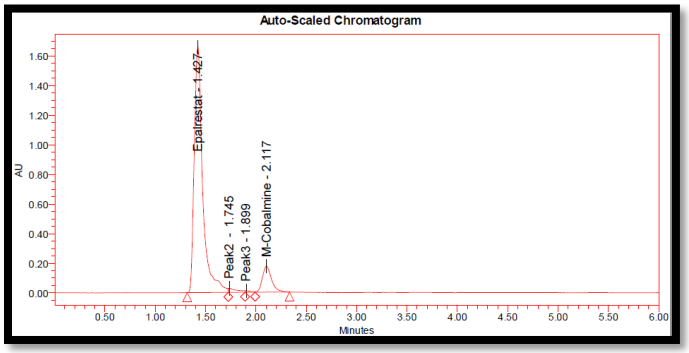


Figure 15: Chromatogram of Photolytic Forced Degradation of Epalrestat and Methylcobalamin

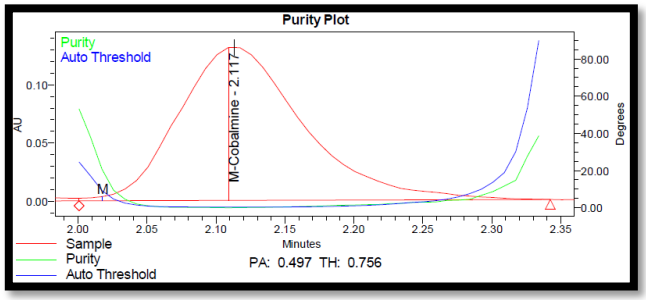


Figure 16: Purity Plots for Epalrestat and Methylcobalamin in Degradation

CONCLUSION

A new Reversed Phase UPLC method described by this manuscript provides a simple, convenient and reproducible approach for the simultaneous estimation and quantification of drugs used Epalrestat and Methylcobalamin in the routine quality control analysis.

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