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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING UPLC-ELSD DETECTION METHOD FOR THE DETERMINATION OF OBETICHOLIC ACID IN BULK AND FINISHED FORMULATIONS

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ABSTRACT

A simple, sensitive and stability indicating reverse phase ultra-performance liquid chromatography with ELSD detection method (RP-UPLC) was developed and validated for the estimation of Obeticholic acid in bulk and finished dosage forms. The drug was subjected to various stress Conditions such as hydrolysis, oxidation, photolytic and thermal degradations to investigate the stability indicating ability of the method. Compound is highly stable against stress conditions. Obeticholic acid is an UV inactive compound having lower UV absorbance and so a ELSD coupled with UPLC system was used for better sensitivity. Efficient chromatographic separation was achieved by using Acquity; UPLC, BEH; C-18; 100 x 2.1mm; 1.7 µm column with the mobile phase consisting of 0.05% Trifluoro acetic acid in water and 0.05% Trifluoro acetic acid in acetonitrile in a gradient elution mode within a short run time of 6.0 minutes at a flow rate of 0.3 ml/min. The developed method was validated as per the current ICH quality guidelines with respect to specificity, precision, accuracy, linearity, robustness and solution suitability. The average recovery values of Obeticholic acid were found to be in the range of 99.99-101.41 %. The developed method was linear with the correlation value of 0.9994 for Obeticholic acid. The repeatability and intermediate precision expressed by RSD were less than 2.0% for Obeticholic acid. The test solution was found to be stable in diluent for 48 h when stored at room temperature. The developed UPLC method is superior in technology against conventional HPLC with respect to speed, resolution, solvent consumption and cost of analysis. This method is compatible to LCMS analysis which enables to identify the unknown impurities or the degradants formed in the process.

KEYWORDS: Obeticholic acid, ELSD (Evaporative Light Scattering Detector), stability indicating UPLC method, Assay validation.

INTRODUCTION

Obeticholic acid belongs to hepatoprotective category. It is a semi-synthetic bile acid, which acts as a farnesoid X receptor agonist and is used for treating primary biliary cholangitis.[1-2] Chemically Obeticholic acid is a dihydroxy-5 beta-cholanic acid, 3 alpha-hydroxy steroid and 7 alpha-hydroxy steroid. It was derived from a chenodeoxycholic acid.[3-5] The key role of the farnesoid X receptor (FXR) as a regulator of bile and cholesterol metabolism in the liver, with preclinical data from numerous studies providing strong rationale for the advancement of FXR agonists as hepatoprotective therapeutics in chronic liver disease.[6-9] The chemical structure of Obeticholic acid was presented in Figure 1. The drug is still not official in any pharmacopoeia.[10] It is known that drugs can undergo physicochemical degradation during manufacturing and storage due to processes like oxidation, reduction, hydrolysis, racemization etc.[11-12] Therefore, understanding drug degradation under stressed conditions is critical in pharmaceutical development because drug stability and degradation products have a significant impact on formulation development, analytical method development, package development, and storage conditions.[13,14] Complete knowledge of API's stability profile is one of the key factors to prevent those risks during manufacturing and storage.[15-17] Thus the study of degradation of drugs should be carried out in stress conditions as recommended by the ICH guidelines. Literature survey revealed that only a few analytical methods were reported for the estimation Obeticholic acid. Bio analytical methods (LC-MS/MS) have been reported for the quantification of Obeticholic acid in biological fluids. Earlier some of analytical journals have been reported on enantiomer separations by chiral methods using HPLC and capillary electrophoresis.[18-21] There are also couple of RP-HPLC methods for estimation of Obeticholic acid using UV and RID

detection with longer run time. The objective of the current work is to develop a stability indicating UPLC-ELSD method with a short run time. The objective is also to develop a stability indicating method by means of performing forced degradation studies on the bulk as well as finished formulations in order to study the impact of excipient on the developed method. The developed method has been validated for all the parameters as per the current ICH quality guidelines. Hence the proposed validated method can be used in quality control laboratories for routine as well as stability analysis of bulk and finished formulations.

Structure of Obeticholic acid

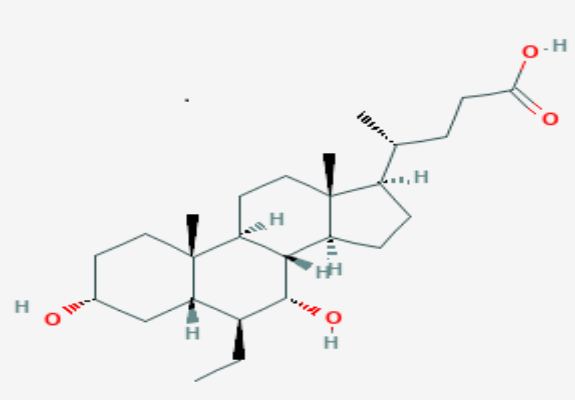

Figure.1: Structure of Obeticholic acid.

MATERIALS AND METHODS

Chemical reagents and sample

Obeticholic acid was a gift sample obtained from a reputed manufacturing unit in Hyderabad, Obeticholic acid tablets (OCALIVA) were purchased from local market in India. Acetonitrile of HPLC grade was purchased from Rankem chemicals (Mumbai, India). Trifluoroacetic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck chemicals (Darmstadt, Germany). HPLC grade water was obtained from milli-Q water purification system (Millipore, Milford, USA).

Equipment

A prominence series Waters Acquity UPLC system equipped with a binary solvent manager pump, an auto sampler, and ELSD detector used for method development, validation and stress degradation studies. The signal output was monitored and processed using Empower-3 software on a Dell computer. Chromatographic separation was achieved on Acquity UPLC BEH C18 100*2.1 mm, with particle size of 1.7 µm. Thermal degradation study was carried out in a hot air oven (Vision lab Equipments) and photolytic degradation was carried out on photo stability chamber purchased from Thermo lab scientific instruments.

Chromatographic conditions

The objective of the present study is to develop a rapid stability indicating UPLC method for the estimation of Obeticholic acid with proper peak shape and resolution. Chromatographic separation was performed on Waters UPLC with Acquity UPLC BEH C18 100*2.1 mm, 1.7 µm column. Mobile phase A was 0.05% Trifluoro acetic acid in water and mobile phase B was 0.05% Trifluoro acetic acid in acetonitrile. Diluent was prepared by mixing water and acetonitrile in the ratio of 50:50 (v/v). Injection volume was 1.0 µl with a flow rate of 0.3 ml/min and analysis was carried out using ELSD detector with a data acquisition time of 6.0 min.

Preparation of buffer-A: Dissolved accurately 0.5 ml of Trifluoro acetic acid in 1000 ml of milli-q water and mixed well. This solution was sonicated and degassed to remove dissolved particles.

Preparation of buffer-B: Dissolved accurately 0.5 ml of Trifluoro acetic acid in 1000 ml of Acetonitrile, sonicated and mixed well.

Preparation of standard solution: A working standard stock solution of Obeticholic acid was prepared by dissolving standard equivalent to 100 mg of Obeticholic acid into 100 ml volumetric flask, to this added 60 ml of diluent and sonicated for 5 minutes and then diluted to the volume with diluent to have a solution concentration of 1000 ppm.

Preparation of diluted standard

Diluted 1ml of the standard stock solution to 100 ml with diluent and mixed well, further diluted 2 ml of the resulting solution to 20 ml with diluent. The obtained solution is of 1.0 ppm.

Preparation of sample solution: Transfer 100 mg of Obeticholic acid sample into 100 ml volumetric flask and added 60 mL of diluent and sonicated in ultrasonic bath for 20 minutes with intermediate shaking and diluted to the volume with diluent. Filter the solution through 0.45 µm nylon membrane filter by discarding 4 ml of filtrate and injected the same solution (1.0 mg/ml).

METHOD VALIDATION

Specificity

Specificity is the ability of the method to measure the analyte response in presence of its potential impurities. Specificity of the developed method was carried out in the presence of blank for the accurate measurement of amount of compound present in the sample. As a part of specificity, stress studies were carried out for Obeticholic acid drug substance, drug product and placebo under stress conditions like oxidation, acid, base, photolytic and thermal (120°C). These stress samples were analyzed using the proposed method at a test concentration of 1000 ppm by using ELSD-UPLC system.

Precision

Precision of the analytical method is the closeness agreement for a series of measurement from multiple samplings. As per the quality guidelines, system

precision, method precision and intermediate precision were analyzed on the homogeneous sample and the % RSD of OBT for precision and intermediate precision was calculated and reported.

LOQ

The quantification limit (LOQ) for Obeticholic acid was established by means of linearity method. The compound solutions from concentration ranging from 0.01 ppm to 0.3 ppm with 5 different levels were prepared and injected. Based on the compound response and STEYX value, the least concentration of compound up to which it can be identified and quantified were calculated and verified.

Linearity

Linearity of the detector response was established for Obeticholic acid with concentration ranging from LOQ to 150 % of the specification level with respect to test concentration. The samples were analyzed as per the described test method. A linearity graph was plotted between the responses of compound (Y-axis) against actual concentration in ppm (X-axis) and determined the correlation co-efficient and Y-intercept at 100 % response.

Accuracy

Accuracy of the analytical method is the closeness of agreement between the true value and experimental value. Accuracy of the compound was performed at 4 different levels ranging from LOQ to 150 % of the specification level of the compound with respect to test concentration level. The % recovery was calculated by comparing the compound level at each level of spiked sample with as such sample.

Robustness

The robustness of the method was evaluated to establish the capability of the method by changing the experimental conditions and studying its impact on the system suitability. Robustness was performed by changing the method parameters like mobile phase flow rate and column temperature.

Solution stability

Solution stability was carried out by storing the standard solution of Obeticholic acid 1000 ppm at room temperature up to 48 hours. This solution was injected at an interval of 0, 24, and 48 hours. The assay content and system suitability results were checked at each time interval.

RESULTS AND DISCUSSION

Method development and Optimization

There was no stability indicating method reported by UPLC-ELSD for the determination and quantification of Obeticholic acid in bulk and finished product. The intention of the method was to quantify of the compound and separate all the potential impurity peaks originate during the forced degradation study and stability studies with proper peak shape and resolution. Forced degradation sample was taken as reference, for the optimization of the UPLC method. Trials were taken by varying the pH value of the mobile phase buffer from 5.0 to 2.0. Finally Trifluoro acetic acid buffer was used based on the LC-MS compatibility.

Initially, the peak shape was broad and long run time using isocratic mode. In order to shorten the run time, gradient separation mode was optimized with good peak shape and elution. Optimal separation was attained on Acquity UPLC BEH C18 column with dimensions 100*2.1 mm, 1.7 µm. Gradient elution was executed using the combination of 0.05% Trifluoro acetic acid in milli-q water buffer (pH~ 1.9) and 0.05% Trifluoro acetic acid in acetonitrile as organic modifier at a flow rate of 0.3 mL/min. UPLC detection was carried in ELSD. Gradient program was cited in Table-1.

Tab. 1: Gradient Program for UPLC method.

Time (minutes)	Flow rate (ml/min)	% of mobile phase-A	% of mobile phase-B
0.0	0.3	50	50
4.0	0.3	10	90
8.0	0.3	10	90
8.1	0.3	50	50

System suitability

System suitability solution was prepared by assay concentration of standard at the specification level (1.0 mg/ml) and injected to evaluate the system suitability of the method and found that Obeticholic acid retention time of 3.48 minutes. Chromatogram of OBT was illustrated in fig-2. The system suitability results were given in table-2. The developed UPLC method was found to be specific for Obeticholic acid in the proposed method. Standard solutions of Obeticholic acid working standard was prepared as per procedure and were injected five times into the HPLC system. The system suitability parameters were evaluated from standard chromatogram. The % RSD for area count and retention time, tailing factor and theoretical plates from five replicate injections are within range and results were shown in Table 2 and Figure 2.

Tab. 2: System suitability results (RT-Retention time, RRT-relative retention time).

Compound Name	RT	RRT	USP Tailing	USP Plate count
OBT	3.48	1.00	1.2	1092756.18

OBT	System suitability
No of Inj's	Area
Inj-1	2799586
Inj-2	2800197
Inj-3	2810395
Inj-4	2798621
Inj-5	2790165
Average	2799792.80
SD	7188.19
% RSD	**0.26**

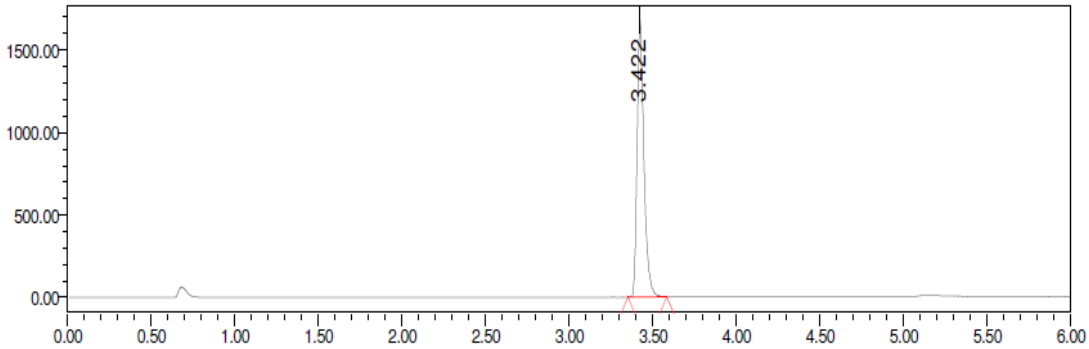

Fig. 2: System suitability chromatogram.

Precision

The precision of a method determines the closeness of agreement between a series of measurements of the same sample. The intraday and interday precisions were carried out 5 times at concentration of 1.0 mg/mL and the %RSD were found to be 0.23 to 1.66%, respectively. The precision result were within the accepted limits of ≤ 2.0 % RSD which proves that the method was precise. The results were tabulated in Table-3. Hence the developed method is precise for its intended use.

Tab. 3: Method precision and intermediate precision data.

OBT	System precision	Method precision	Ruggudness-1	Ruggudness-2
No of Injs	**Area**	**Area**	**Area**	**Area**
Inj-1	2824591	2879322	2869334	2811031
Inj-2	2810540	2875489	2815647	2769841
Inj-3	2809745	2859841	2792310	2793461
Inj-4	2809915	2857660	2806125	2699874
Inj-5	2811632	2867324	2890216	2809314
Average	2813284.60	2867927.20	2834726.40	2776704.20
SD	6363.60	9462.93	42596.94	46023.10
% RSD	**0.23**	**0.33**	**1.50**	**1.66**

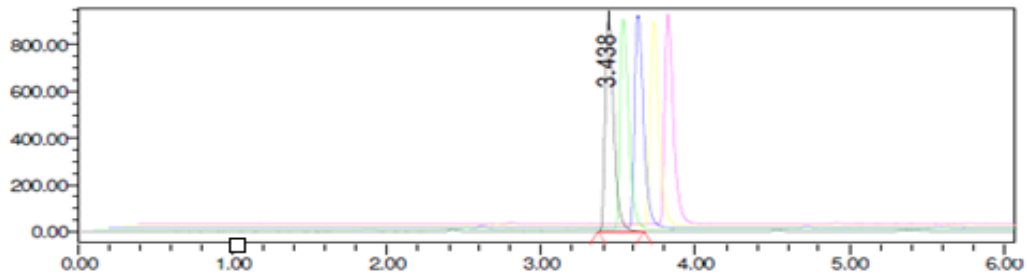

Fig.3. Precision chromatogram overlay.

LOD and LOQ

LOD is a limit test parameter and it is a test to determine whether the analyte concentration was present within the specification limit or not. LOQ is a parameter for quantitative assay used particularly for determination of impurities or degradation products as it used for minimum concentrations of analyte in sample. The LOD and LOQ were found to be 0.03 and 0.1 respectively and the % RSD for LOQ precision of OBT was 3.77%, which proves the method was sensitive. The LOQ chromatogram was shown in Fig-4 and the LOQ precision values were mentioned in Table.4.

Tab. 4: LOQ precision establishment for Obeticholic acid.

OBT	
No of Injs	**Area**
Inj-1	78923
Inj-2	77924
Inj-3	74244
Inj-4	78870
Inj-5	72662
Average	76524.60
SD	2886.71
% RSD	**3.77**

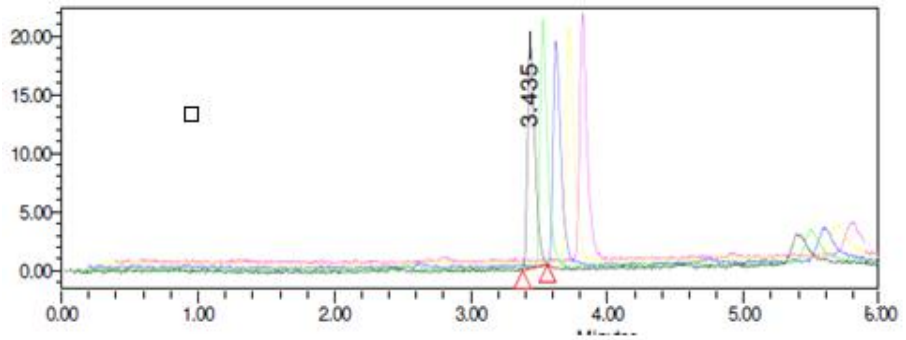

Fig. 4: LOQ chromatogram.

Linearity and range

Linearity of the developed method was evaluated for six different levels of Obeticholic acid assay. The concentrations ranged from LOQ to 150 % of assay specification limit. The respective peak area was recorded and plotted against standard concentration and the graph resulted in straight line. The correlation coefficient, slope, intercept and % Y-intercept values were calculated and tabulated for Obeticholic acid. The compiled results were tabulated below in table-5. Correlation Co-efficient was found to be 0.9994 and the linearity graph shown in Fig.5.

Tab. 5: Linearity results for Obeticholic acid.

S.No	Concentration (%)	Area response of OBT
1	0.010	76634
2	50.00	1399765
3	75.00	2108451
4	100.00	2799793
5	125.00	3501954
6	150.00	4258741.0
Correlation coefficient		**0.9994**

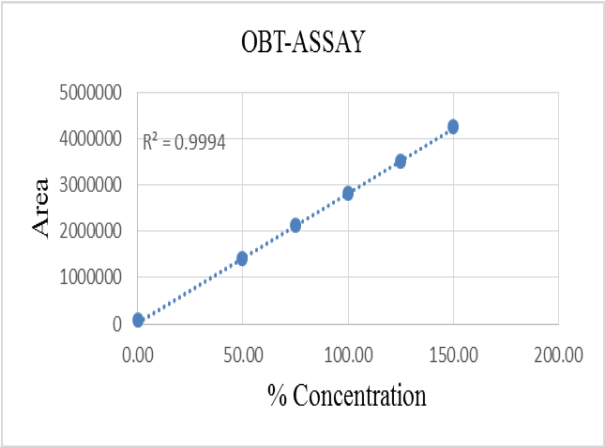

Fig. 5: Linearity graph for OBT assay.

Accuracy

Recovery studies were performed to judge the accuracy of the test method. The study was evaluated by spiking the known quantity of OBT at various levels on the placebo. From the amount of OBT found the % recovery was calculated. Recovery was performed at four different levels ranging from LOQ to 150 % of the specification level. The % recovery of OBT was found to be within the acceptance criteria of 98.0% to 102.0%. So the method is accurate for the determination of Obeticholic acid quantification. Good recovery results obtained for the developed method indicates that this method can be used for regular quality control assay test for Obeticholic acid. The mean recovery values for the OBT assay were tabulated in table-6.

Tab. 6: Accuracy results for Obeticholic acid.

S.No	Conc level	% Mean recovery ± SD of OBT
1	LOQ	100.26
2	50%	99.99
3	100%	100.00
4	150%	101.41

Robustness

Robustness of the method was performed by changing flow rate (± 0.1mL/min) and change in Organic ratio (± 1.0%). The results were summarized in Table 7. It was observed that even in minor changes of method conditions there was no marked changes in the results demonstrate that the method developed was robust. The robustness results were within the accepted limits of ≤ 2 % RSD.

Tab. 7: Robustness results for Obeticholic acid.

Robustness					
OBT	**As such conditions**	**Flow Decrease**	**Flow Increase**	**Organic Decrease**	**Organic Increase**
No of Injs	Area	Area	Area	Area	Area
Inj-1	2811245	2799587	2786981	2799301	2828813
Inj-2	2809754	2798863	2790369	2792087	2819745
Inj-3	2810911	2826974	2800630	2788397	2802295
Inj-4	2798654	2798431	2801227	2800653	2811963
Inj-5	2786548	2816978	2810241	2802765	2820067
Average	2803422.40	2808166.60	2797889.60	2796640.60	2816576.60
SD	10780.12	13098.74	9310.77	6111.01	9964.95
% RSD	**0.38**	**0.47**	**0.33**	**0.22**	**0.35**

Solution stability

There was no change in the area counts of the Obeticholic acid, when both the standard and sample solutions were monitored periodically for a period of 24 Hrs at room temperature (not more than 27 °C) and at a temperature of 5 ± 3°C. It was observed that the standard solution is stable for 24 hrs at 5 ± 3°C and sample solution is stable for 8 hrs at 5 ± 3°C. The solution stability values for the OBT assay were tabulated in table-8.

Tab. 8: Solution stability results for Obeticholic acid.

Solution stability			
OBT	**0hrs**	**24hrs**	**48hrs**
No of Injs	**Area**	**Area**	**Area**
Inj-1	2799586	2810074	2799632
Inj-2	2800197	2809365	2806211
Inj-3	2810395	2809741	2806574
Inj-4	2798621	2816573	2826540
Inj-5	2790165	2799657	2796851
Average	2799792.80	2809082.00	2807161.60
SD	7188.19	6050.71	11616.53
% RSD	**0.26**	**0.22**	**0.41**

Specificity

The specificity of the method was evaluated by verifying the mass balance and assay of the sample. The method was found to be specific as there was no interference from blank and placebo at the retention time of main peak. No degradants peaks were observed at the retention time of Obeticholic acid during the forced degradation and stability study indicates that the method is stability indicating and also good recovery of Obeticholic acid assay. Typical chromatograms of (a) blank (b) placebo (c) standard and (d) sample were mentioned in figure-6.

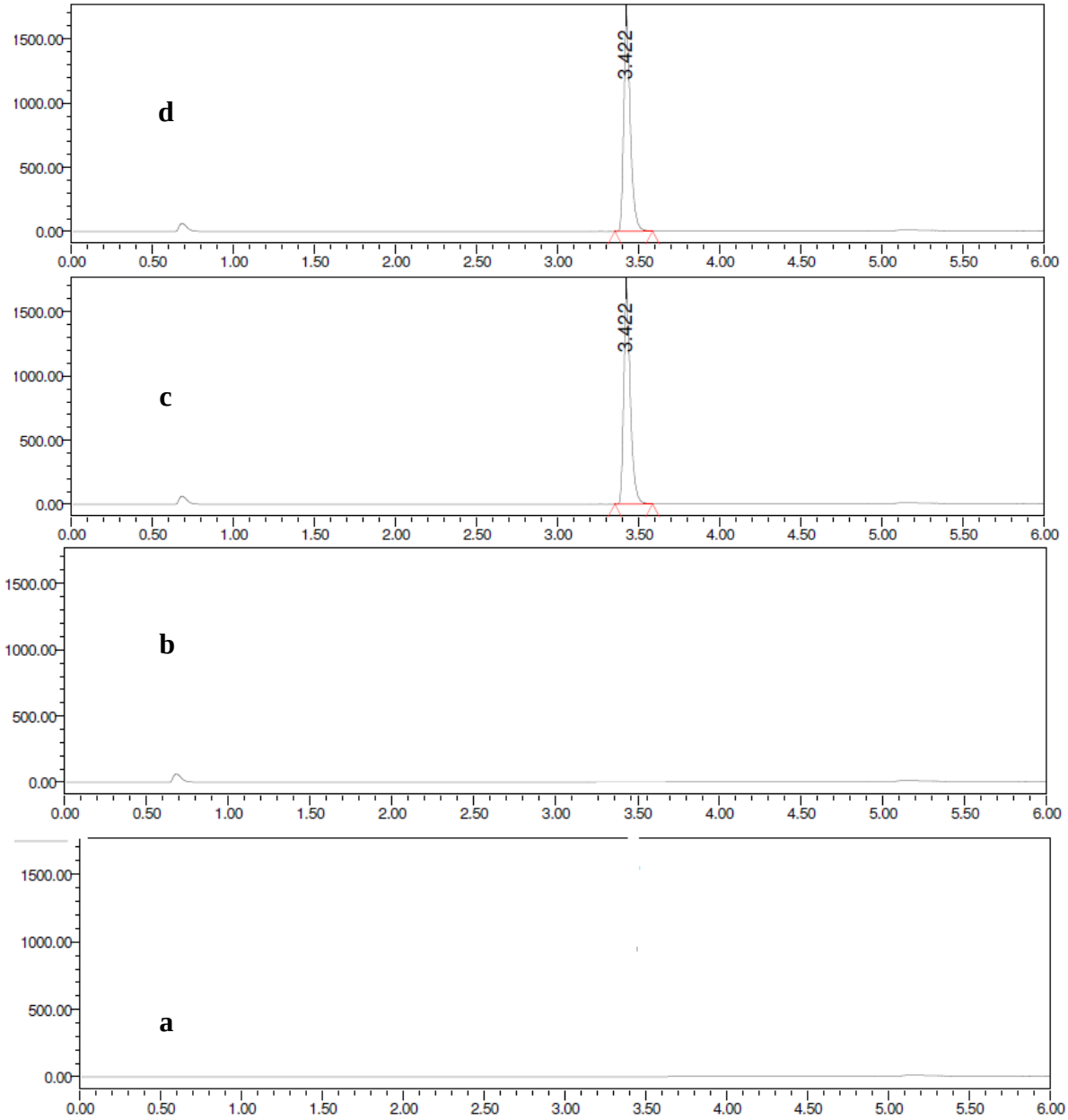

Fig.6. Typical chromatograms of (a) blank (b) placebo (c) standard and (d) sample.

Forced degradation studies

Forced degradation studies were performed to establish the stability indicating power of the method. In this study Obeticholic acid and placebo were subjected to acidic, basic, peroxide, thermal and photolytic stress studies on sample concentration of 1.0 mg/ml in diluent. Sample equivalent to 100 mg of Obeticholic acid was placed into 100 ml volumetric flask added 60 ml of diluent and sonicated for 10 min with intermediate shaking and then added respective degradant (Acid, Alkali, Oxidant) and performed the stress study. Samples were neutralized after degradation and then diluted to the volume with diluent and injected to verify the stability indicating power of the analytical method. Stress study results were tabulated in table-9. The chromatograms for forced degradation study were summarized figure-7.

Tab. 9: Degradation studies for Obeticholic acid.

S.No	Stress condition	% Assay Drug remained	% impurities
1	5 mL 5N HNO3/5h,60°C	99.90	No degradation
2	5 mL 3N NaOH/10h,60°C	100.28	No degradation
3	5 mL 10% H2O2/24h, 60°C	100.43	No degradation
4	120 °C_48 Hrs	98.95	No degradation
5	Photolytic stability(UV)	99.38	No degradation

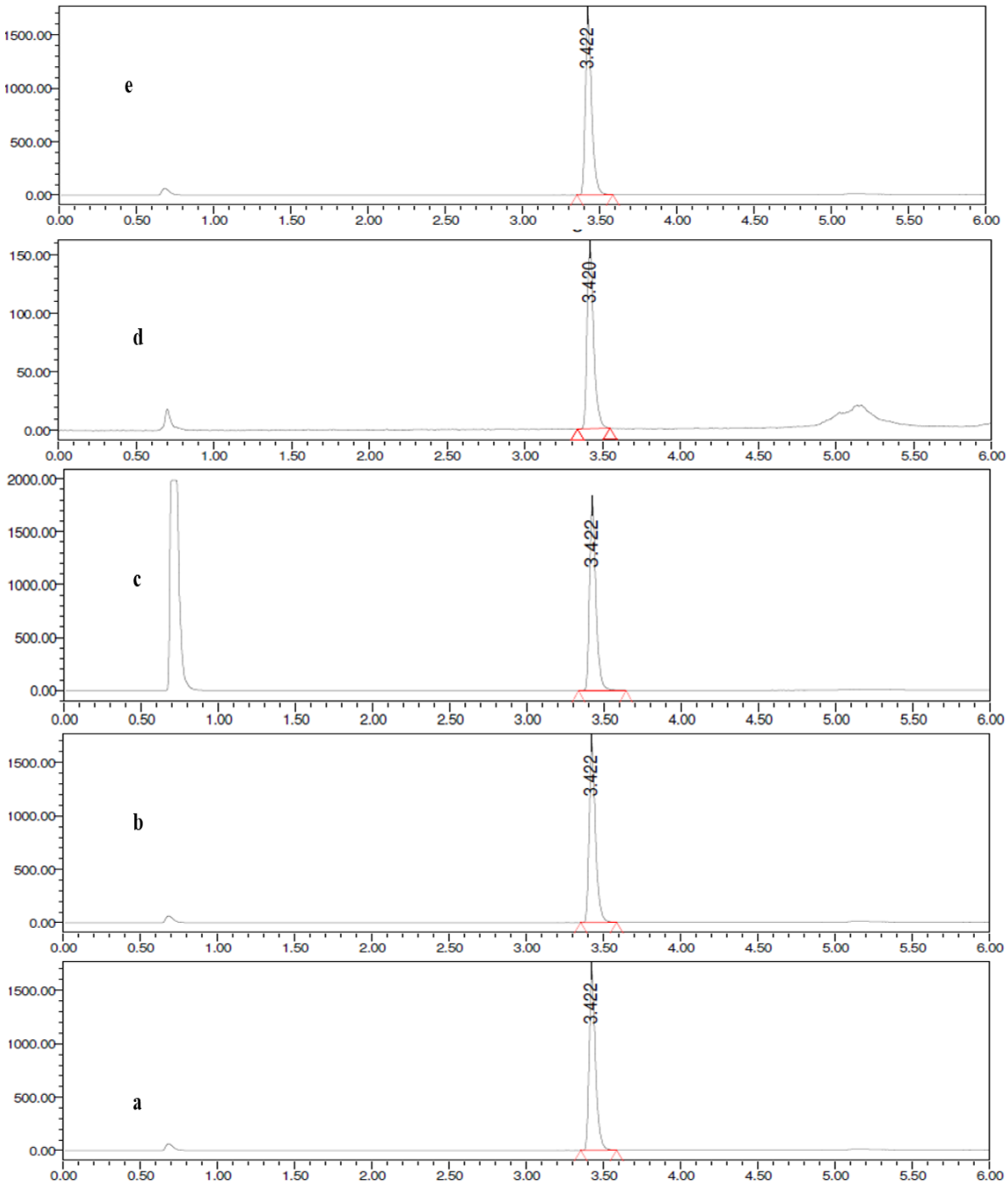

Fig. 7: Typical degradation chromatograms of (a) UV (b) Thermal (c) Oxidative, (d) base and (e) Acidic conditions.

CONCLUSION

A novel, reverse phase UPLC method with ELSD detector has been developed and validated for the quantification of Obeticholic acid in bulk and its pharmaceutical dosage form. The proposed UPLC method obeys linearity within the concentration range of 0.01-150% for OBT assay with correlation coefficient of not less than 0.999. LOD and LOQ values are 0.03 and 0.011 ppm respectively. Inter and intraday precision with cumulative % RSD for OBT assay were found to be 0.23% to 1.66% respectively. % Recovery values for the OBT assay were found to be between 99.9% and 101.4% for the concentration range between LOQ and 150% of the test concentration (1.0 mg/ml). The method is found to be specific and there is no interference of degradation impurities with OBT peak. The developed method was validated as per ICH guidelines. All the validation parameters were found to be well within the acceptance criteria. We concluded that the method is accurate, precise, linear and robust. The developed method can be successfully applied for the analysis of Obeticholic acid bulk and pharmaceutical dosage form in quality control laboratories.

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