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A VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR QUANTIFICATION OF OBETICHOLIC ACID IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

An appropriate, well-thought-out approach was developed for the assessment of obeticholic acid using the RP-HPLC method. The conditions for the chromatographic analysis were as follows: the column was a Kromasil C18 (150mm*4.6mm5m) with an adaptable sorting of 0.01N Kh2po4: Acetonitrile in a ratio of 70:30, the flow rate was maintained at 1.0 ml/min, the irrefutable proof resolution was 260.0 nm, the temperature of the mixture was maintained at 30oC, and the diluent was of effective direction. As an extra way, conditions were set up. The standard dissimilar times and outcomes were well beneath the affirmation rules, which concentrated the system's sensitivity limitations. R2 was 0.999, and linearity was considered between 25% and 15%. Repeatability was assessed as 1.0 and focal point of the street exactness as 0.9 in terms of precision. Separately, the limits of detection (LOD) are 0.05 g/ml and 0.17 g/ml. Using an out-of-process review of the front line, a decision-making rate of 100.07% was achieved. In all cases, the flawlessness edge was greater than the ethical point and inside the agreeable reach, and debasement assessments of Obeticholic uncomfortable were bundled up. A full-scale strategy was not implemented; nonetheless, this technique can be used for the evaluation of Obeticholic harm plans in the future.

KEYWORDS: HPLC Obeticholic acid, Method development. ICH Guidelines.

INTRODUCTION

Obeticholic acid is a semisynthetic analogue of the chenodeoxycholic acid, a hydrophobic primary bile acid, the natural farnesoid X receptor (FXR) agonist in humans produced in the liver from cholesterol. It is sold under the brand name of OCALIVA.[1] Chemically, it is (4R)-4-[(3R,5S,6R,7R,8S,9S,10S,13R,14S,17R)-6-ethyl-3,7-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoic acid, having a molecular formula of $C_{26}H_{44}O_4$, its Chemical structure is shown figure 1. It is solid white to beige powder soluble in methanol, DMSO and ethyl alcohol, insoluble in water.

It is used to treat several liver diseases. It was accepted as an orphan drug built on its reduction in the level of the biomarker alkaline phosphatase as a surrogate endpoint for clinical benefit. Obeticholic acid has been approved in combination with ursodeoxycholic acid to treat primary biliary cholangitis for the patients who are intolerant or not responded effectively to ursodeoxycholic acid.[2] It given orally, binds to the farnesoid X receptor (FXR), found in the nucleus of cells in the liver and intestine, which is a key regulator of bile acid metabolism. Obeticholic acid increases bile flow from the liver and suppresses bile acid formation, thus decreasing the contact of the liver to toxic levels of bile acids.[3, 4]

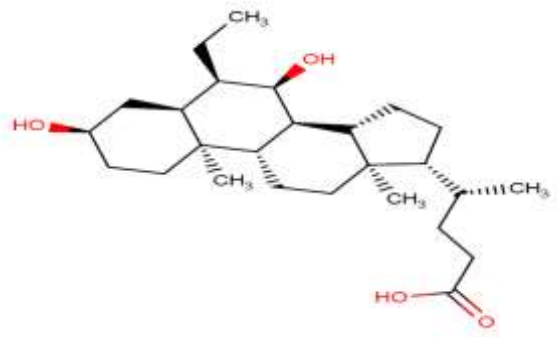

Fig. 1: Structure of Obeticholic acid.

Obeticholic acid can be detected by several techniques such as NMR, LCMS-MS[5-8], although the gas and liquid chromatographic methods have been realized as the most suitable**.** HPLC technique using UV and electrospray ionization-mass spectrometry (ESI-MS) detection was established for the separation and determination of obeticholic acid (OBE) and its related compounds.[9] Detection and Evaluation of process related impurities in Obeticholic Acid Drug material employing HPLC Method.[10] A part of these, various analytical methods were advertised for the determination of obeticholic acid in bulk and

pharmaceutical formulations.[11-17] Based on above Literature survey, there are few methods for the Estimation of Obeticholic acid. Therefore, an attempt was made to develop and validate a simple and economical liquid chromatographic method for the quantification of Obeticholic acid. In present research work, RP-HPLC Method for the quantification of the Obeticholic acid in pharmaceutical formulation has been established and validated using reversed phase high performance liquid chromatography method.

MATERIALS AND METHODS

Chemicals and reagents

Obeticholic acid pure drug was obtained as a contribution sample from Hetero drugs Pvt. Ltd, Hyderabad, India. Methanol, Acetonitrile and purified water of HPLC grade and Glacial Acetic acid, Phosphate buffer, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid of A.R grade were purchased from Rankem (India) Ltd. Bhiwandi, Mumbai, India. The 0.45µ nylon filters were purchased from Millipore.

Instruments

The HPLC system (waters) auto sampler separation module HPLC 29765 consisted of a high-pressure pump and 10 µL capacity injector loops. The system was well equipped with empower 2 software for monitoring and processing of data. The analytical column used was STD Kromasil C18 (150mm x 4.6mm; 5µm). UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was used for measuring absorbance. Other equipment like Micro balance analytical balance (Sartorius) was used for weighing of the materials. P^H meter (Thermo scientific) Degassing of the mobile phase was done by ultrasonic bath Sonicator.

Chromatographic Condition

The mobile phase comprised of 0.01N KH_2PO_4: Acetonitrile in a proportion of 70:30 v/v. Samples were analysed using the following parameters. Flow rate; 1ml/min, injection volume; 10 ml, run time; 10 min, column oven temperature; 30^oc and detection wavelength; 260.0 nm.

Method Development

Any analytical method started using reverse phase liquid chromatography as it is commonly used, and C-18 columns are also available. RP-HPLC method was developed for the estimation of Obeticholic acid content in formulation. The mobile phase was selected based

on the sensitivity of the process, the time necessary for the analysis, easily available solvents and simplicity of preparation. The mobile phase was premixed and filtered through a 0.45µm filter and sonicated for 10min to remove gases. Optimization of the mobile phase was taken based on various parameters such as retention time, the number of theoretical plates and resolution.

Solutions preparation

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and buffer taken in the ratio of 50:50.

Preparation of Standard stock solution (100µg/ml)**:** 2.5mg of accurately weighed Obeticholic acid was dissolved in 3/4th of diluent, sonicated for 10 minutes and volume was made up to 25ml with diluent.

Preparation of Standard solution (10µg/ml**):** 1ml of Obeticholic acid stock solution was pipetted out into a 10ml volumetric flask and made up with diluent.

Preparation of Sample stock solution: (200µg/ml) 20 tablets were weighed, and the average weight of tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 50 ml volumetric flask, diluent was added and sonicated for 25 min, finally the volume was completed and filtered by HPLC filters.

Preparation of Sample solution (10µg/ml**):** 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent.

Preparation of buffer: 0.01N Potassium dihydrogen ortho phosphate

Accurately weighed 1.36gm of Potassium dihydrogen Ortho phosphate was transferred in a 1000ml of Volumetric flask, about 900ml of milli-Q water was added, sonicated for degassing and finally made up the volume then 1ml of Triethylamine was added and PH adjusted to 3.0 with dil. Orthophosphoric acid solution.

Preparation of mobile phase

Acetonitrile and 0.01N KH_2PO_4 were mixed in a ratio of 70:30 v/v. The solution was degassed in an ultrasonic water bath for 5 minutes and filtered through 0.45µm filter under vacuum.

Procedure for method development

A mobile phase of Acetonitrile and phosphate buffers in the percentage of 70:30 % v/v was found to be the most suitable mobile phase for best separation of Obeticholic acid. The mobile phase was pumped all through the STD Kromasil C_{18} column owning a length of 150mm x 4.6mm; and 5µm particle size with a flow rate of 1 ml/min. The column was at a 30^0c. The column was equilibrated by pumping the mobile phase for at least 30 minutes prior to the injection of drug solution.10µl of standard and sample solutions were loaded in six replicates separately in injection port of the instrument and the area for the drug peak was measured. The detection of drug peak was monitored at 260 nm. The run time was set at 10 min. Under these optimized chromatographic conditions, the retention time for the Obeticholic acid was 3.333min. The typical chromatograms of both standard and sample solutions are given in figures 3 and 4. From the peak, the content of the above drug in ppm was calculated using the following formula.

$$\text{Assay} = \frac{\text{Sample peak area}}{\text{Standard Peak area}} \times \frac{\text{Standard Dil. Factor}}{\text{Sample Dilution Factor}} \times \frac{\text{Avg. Wt of Tab}}{\text{Labelled Claim}} \times \text{potency of standard}$$

Stress Degradation Studies[18]

The stability of analytical method was determined by conducting Degradation studies. Standard solution was stressed at different strain conditions includes acid (1N Hydrochloric acid at 60^0c for 30min), alkali (1N Sodium hydroxide at 60^0c for 30min) and neutral hydrolysis (water at 60^0c for 30min), oxidative (20% hydrogen peroxide at 60^0c for 30min), thermal (105^0c for 6 hr), photo stability (exposure to UV light for 7days) and Relative humidity (60^0c,75%RHfor 30min), neutral (water at a 60ºc for 6hrs).The assay of the degraded injected samples(10ppm) was calculated.

Method Validation[19]

The validation of analytical technique confirms that the characteristics of the method if they fulfil the requirements of the method. The Projected method was validated agreeing to ICH guidelines for system suitability, specificity, linearity, accuracy, and precision, limit of detection and quantification and robustness.

RESULTS AND DISCUSSION

Assay of Marketed Formulation

The chromatograms of both Standard dilution of pure drug and sample solution comprising 10µg/ml were recorded, the mean retention time of Obeticholic acid was found to be 2.294 and the mean percentage purity of drug was calculated. The results are shown in Table 1 and respective chromatograms are depicted in Figure 2 and 3.

Table 1: Assay results of Obeticholic acid formulation.

Sample No	%Assay
1	98.41
2	98.47
3.	98.29
4.	99.11
5.	100.36
6.	99.77
AVG	99.07
STDEV	0.84
%RSD	0.9

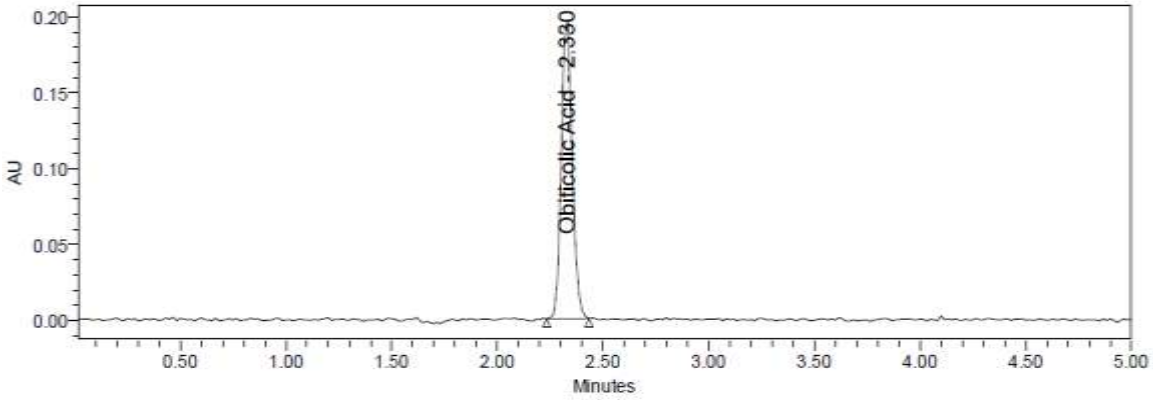

Fig. 2: Typical Chromatogram of Obeticholic acid From Standard solution.

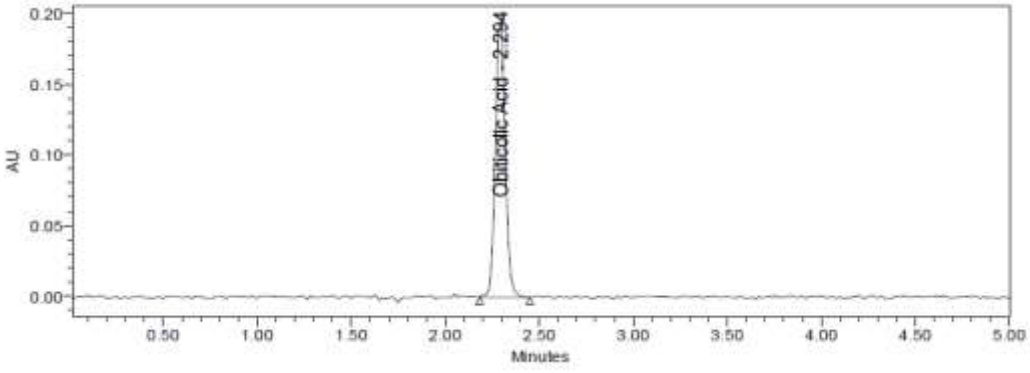

Fig:3 Fig. 3 Typical Chromatogram of Obeticholic acid From Sample Solution.

Method Validation

System suitability: After equilibration of column with mobile phase, the blank preparation (twice) and five replicate injections of 10ppm of Obeticholic standard solution were injected through an auto injector into the optimized chromatography process and the chromatograms were documented. The system suitability parameters were measured, and the results are shown in Table 2 and the chromatogram is revealed in Fig.2. The results have been demonstrated with respect to %RSD, USP plate count and USP peak tailing. The %RSD for the five replicate injections was less than 2%.

Table 2: System suitability results.

Injection No	RT (min)	Peak Area	USP Plate count	USP tailing
1	2.304	715087	9534	1.15
2	2.307	711530	10300	1.14
3	2.313	703776	9551	1.07
4	2.315	714690	9845	1.07
5	2.330	700687	9508	1.07
6	2.333	700029	10052	1.09
Mean		**707633**		
Std.Dev.		**6949.7**		
%RSD		**1.0**		

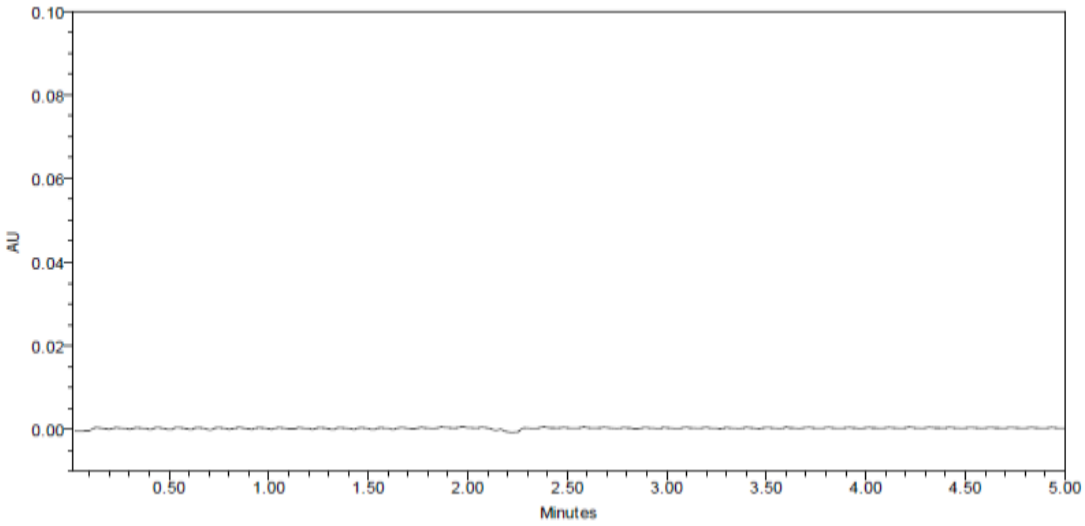

Fig. 4: A typical Chromatogram of blank.

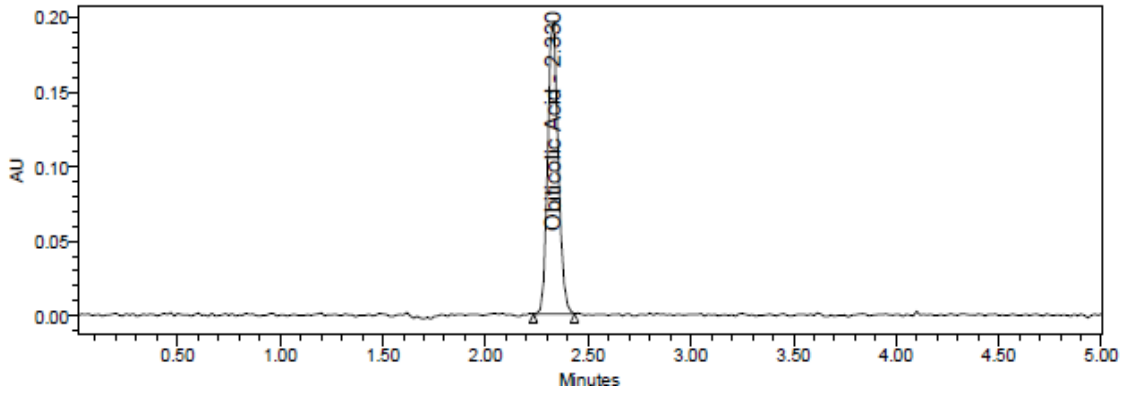

Fig. 5: A typical Chromatogram of system suitability.

Specificity: The specificity of the analytical process was decided by injecting a blank (mobile phase), Obeticholic acid and sample preparations into the chromatography system in the same experimental circumstances. The chromatograms were judged. The retention time for both standard and sample were identical i.e. 2.330 min. it was observed from the chromatogram of blank that there was no interference at the retention time of Obeticholic acid peak. Chromatograms are shown from Fig.3-5.

Linearity: The linearity was assessed by measuring the peak area response of all solutions over the concentration range of the 2.5 ppm to 15 ppm. Six concentrations were injected in replicate throughout the range. A linear correlation was attained between the peak response and concentration of Obeticholic acid for LC method as proven in Fig.6 and was confirmed by the value of regression coefficient (R^2 = 0.9996). The values are shown in Table 3.

Table 3: Linearity data for Obeticholic acid.

Linearity Level (%)	Concentration (ppm)	Peak response
25	2.5	200139
50	5	360960
75	7.5	533730
100	10	705308
125	12.5	881317
150	15	1052961
Slope	**69508**	
y-intercept	**121750**	
R^2	**0.9996**	

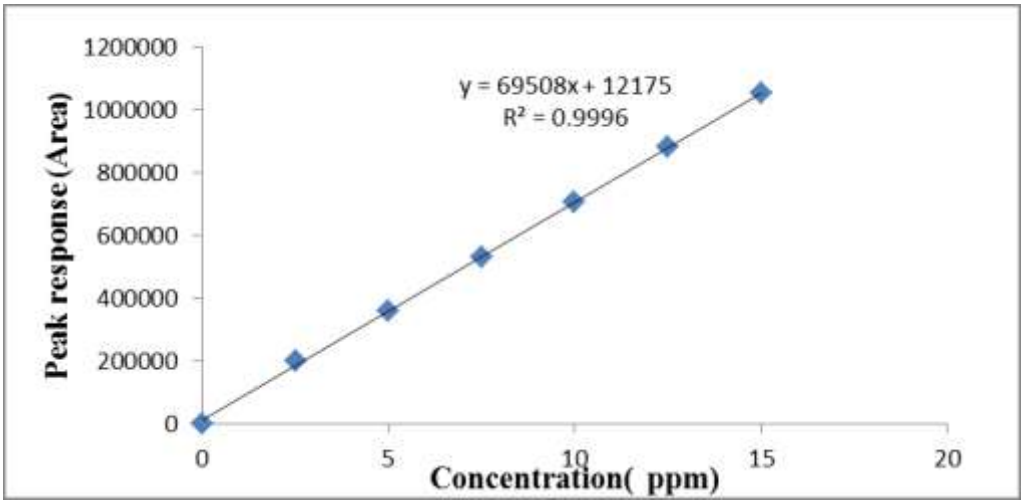

Fig. 6: Typical Calibration curve for Obeticholic acid.

Accuracy (Recovery Studies) The Accuracy of the method was calculated through recovery experiments and was performed by spiking known amount of Obeticholic acid at Quantitation limit, 50%, 100%, and 150% of 10ppm in Sample Preparation. Each level was analysed in triplicate and % recovery was calculated. The recovery was found to be in between 99.57% and 100.65% and the results are summarized in Table 4.

Table 4: Accuracy data for Obeticholic acid.

Accuracy Level %	Amount Spiked (µg/mL)	Amount recovered(µg/mL)	% Recovery	Mean %Recovery
50	50	4.97	99.47	100.07
50	50	5.02	100.37	
50	50	5.01	100.24	
100	100	10.06	100.57	
100	100	10.13	101.26	
100	100	10.06	100.65	
150	150	15.02	100.15	
150	150	14.94	99.57	
150	150	14.75	99.35	

Precision: Repeatability (Intra-day precision) and intermediate (inter-day) study were evaluated by spiking six repeated injections of sample solution of 10ppm. The peak responses of Obeticholic acid were measured and % relative standard deviation of was found to be less than 2. The results are summarized in the Table 5.

Table 5: Precision data for Obeticholic acid.

Preparation No	Intra-day Peak Area	Inter-day Peak area
1	699186	697463
2	699614	698179
3	698347	710081
4	704157	701434
5	713058	707062
6	708830	703382
Mean	**703865**	**703382**
Std. Dev	**5994.3**	**5129.4**
%RSD	**0.9**	**0.7**

Limit of Detection and Quantitation: The detection and quantitation limits of Obeticholic acid were determined by the signal-to-noise ratio (S/N) method. This ratio for LOD is 3:1 and LOQ is 10:1. Solution of Obeticholic acid was prepared around its quantitation limit (QL) concentration and injected in six replicates. The Ditection and quantification limits of the Obeticholic acid in this method are found to be 0.05µg/ml and 0.17µg/ml. The chromatograms are in Fig.7 and 8.

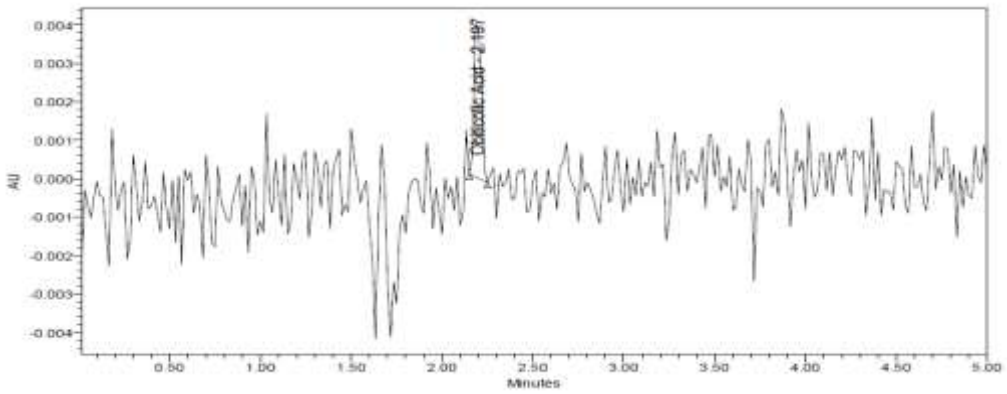

Fig. 7: LOD Chromatogram of Obeticholic acid.

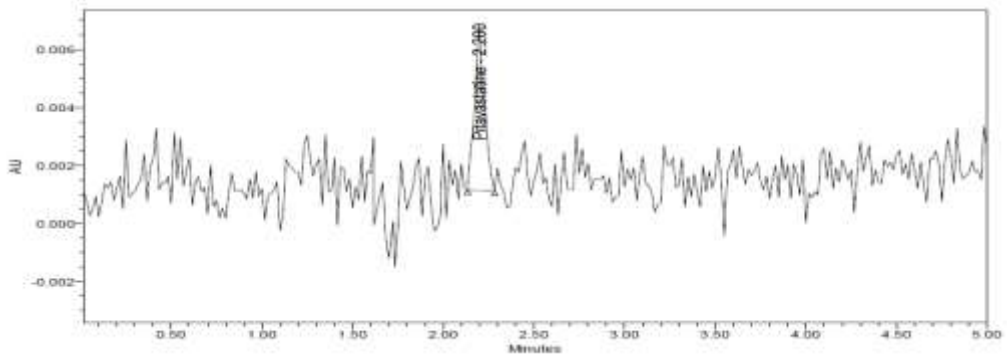

Fig. 8 LOQ Chromatogram of Obeticholic acid.

Robustness: Robustness of the method were established by finding the retention time of Obeticholic acid under minor but purposely modified chromatographic conditions like flow rate, mobile phase composition, wavelength and column temperature on lower and higher side of the original values. The %RSD of retention time and response for Obeticholic acid under these customized chromatographic conditions was less than 2.0%. Therefore, the method was robust for determination of Obeticholic acid content and results are summarized in Table 6.

Table 6: Robustness results for Obeticholic acid.

Parameter	RT (min)	%Difference	%RSD
Original Method	2.2	-	-
Flow-0.9ml/min	2.1	0.1	0.8
Flow-1.1ml/min	2.0	0.2	0.3
Mobile phase-75:25 % v/v	2.1	0.1	0.7
Mobile phase-65:35 % v/v	2.3	0.3	0.8
Column Temperature-33^{0}c	2.1	0.1	0.6
Column Temperature-37^{o}c	2.3	0.1	0.4
Wavelength-258 nm	2.2	0.0	0.0
Wavelength-262 nm	2.2	0.0	0.0

Degradation Studies

There was no interference from sample placebo and degradants peaks and Assay of analyte from each stress condition shows that all the samples pass the restrictions of degradation. Various stress conditions and assay results of the analyte are given in Table 7.

Table 7: Stress study results of Obeticholic acid.

Stress condition	% Assay
Normal	99.07
1N HCl at 60^{0}c for 30min	96.08
1N NaOH at 60^{0}c for 30min	97.43
20% H_2O_2 at 60^{0}c for 30min	98.57
At 105^{0}c for 6 hr	98.95
UV light for 7days	99.15
At 60^{0}c,75%RHfor 30min	97.02
H_2O at a 60^{o}c for 6hrs	99.15

CONCLUSION

The RP-HPLC method is accurate, precise, reproducible, specific and stability indicating. The method has been found to be better than previously existed methods because of its wide range of linearity, use of economical and readily available mobile phase, UV detection and low Rt. All these components make this method is suitable for quantification of the analyte in

pharmaceutical dosage forms. So, the method can be productively used for routine analysis of Obeticholic acid in formulations.

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