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The Development and Validation of an Analytical Method for Determination of Related Substances In Ursodeoxycholic Acid Tablets by Liquid Chromatography

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ABSTRACT

To develop rapid, rugged, precise and an accurate stability indicating analytical method for estimation of related substances in Ursodeoxycholic Acid tablets. The separation of impurities and Ursodeoxycholic Acid drug is achieved by an isocratic chromatographic method on C18, 250 mm x 4.6 mm, 5µm column. The mobile phase consists of buffer, acetonitrile and methanol in the ratio of 35:28:37 v/v/v pumped at a flow rate of 1.0 ml/minute 35:37:28v/v/v pumped at a flow rate of 1.0 ml/minute. The detection was carried out by using refractive index (RI) detector. The proposed chromatographic method was validated and found to be linear over the concentration range from LOQ to 150.0% of impurity limit level. Overall mean recovery of Chenodeoxycholic acid impurity was found to be 100.6±4.0%w/w The method was found to be simple, stability indicating, precise, accurate and robust which can be utilized for estimation of related substances in Ursodeoxycholic Acid tablets

Keywords: Ursodeoxycholic Acid, Impurity, chromatography, Forced degradation, stability indicating.

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INTRODUCTION

Ursodeoxycholic acid (UDCA) is a chemical called a bile acid (Figure 1). It occurs naturally in bile and can be used to dissolve gallstones. The liver produces bile, which is then stored in the gall bladder. Bile is released by the gall bladder to aid the digestion of fats. UDCA the 7beta-epimer of chenodeoxycholic acid has multiple hepatoprotective activities. UDCA modifies the bile acid pool, decreasing levels of endogenous, hydrophobic bile acids while increasing the proportion of nontoxic hydrophilic bile acids. UDCA has a choleretic effect, increasing hepatocellular bile acid excretion, as well as cytoprotective, antiapoptotic, and immunomodulatory properties. UDCA has been shown to delay development of gastroesophageal varices and progression to cirrhosis as well as to improve long-term survival in patients with primary biliary cirrhosis. Significant improvement of abnormal liver tests may be achieved during UDCA therapy in patients with primary sclerosing cholangitis, intrahepatic cholestasis of pregnancy, cystic fibrosis-associated liver disease, nonalcoholic fatty liver disease, graft-versus-host disease of the liver, total parenteral nutrition-induced cholestasis, and in some pediatric cholestatic liver diseases. However, unlike the effects of UDCA in primary biliary cirrhosis, the long-term effects of UDCA in disease progression and survival in these other conditions remain to be established [1-3].

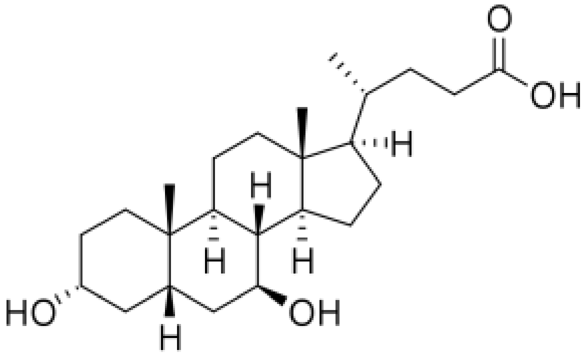

Figure 1 Structure of Ursodeoxycholic Acid

This research was focused on development of a simple, precise, accurate, specific and robust stability indicating reverse phase chromatographic method for quantification of impurities of Ursodeoxycholic Acid in its pharmaceutical dosage forms [4-5]. This method can be used in quality control of manufactured and developed dosage forms. The simplicity of proposed method is economical and can be utilized by common laboratories. The developed method was validated as per International Conference on Harmonization (ICH) Q2(R1) guideline and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <1092> [6-8]. The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38 [9].

MATERIALS AND METHOD

HPLC grade methanol, acetonitrile was purchased from Merck Chemicals, Mumbai, India. Ultrapure water was generated from Milli-Q water purifier.

Instrumentation and Methods

Instrument:

Thermo dionex –Ultimate 3000 High performance liquid chromatography (HPLC) system with RI Detector.

Chromatographic parameters:

The chromatographic column used was a Luna Phenomenox, C18(2) 100 A°, 250 mm x 4.6 mm, 5µm which was maintained at 40º C. The mobile phase was prepared by mixture of buffer, Acetonitrile and methanol of 35:28:37v/v. The flow rate of the mobile phase was 1.0 ml/min. The injection volume was 100.0 µL. The column effluents were monitored by RI detector at sensitivity 256 mV. The detector temperature and column oven temperature was set at 40.0º C.

PREPARATION OF SOLUTIONS

Preparation of buffer solution:

Dissolve 0.78g of sodium dihydrogen orthophosphate dihydrate in 1 L of water. Adjust pH to 3.00 ± 0.05 with orthophosphoric acid (10 % v/v), mix well and filter through 0.45µm nylon membrane filter paper.

Preparation of mobile phase:

Mix buffer, methanol and acetonitrile in the ratio of 35:37:28v/v/v. Mix well and sonicate to degas before use.

Preparation of sample diluent: Use mobile phase as sample diluent.

Preparation of blank solution:

Dilute 5 mL of methanol to 50 mL with sample diluent in a 50 mL volumetric flask and mix well.

Standard stock solution:

Transfer an accurately weighed quantity of about 22.5 mg of UDCAC RS/WS into a 50 mL volumetric flask. Dissolve in about 20 mL methanol and dilute to volume with same.

Standard solution:

Dilute 5.0 mL of calibration stock solution to 50.0 mL with sample diluent in 50 mL volumetric flask. Mix well and filter through 0.45 µm PTFE syringe filter discarding first few mL.

Sample solution:

Weigh and triturate 20 tablets to fine powder. Transfer an accurately weighed quantity of powder sample equivalent to about 450 mg of UDCA into a 50mL clean and dry volumetric flask. To this add 5.0 mL of methanol, sonicate for 5 minutes with intermittent shaking, allow solution to cool to room temperature and make to volume with sample diluent and mix well. Centrifuge at 3000 rpm for 5 minutes. Collect the clear supernatant and filter through 0.45 µm PTFE syringe filter discarding first 3mL of filtrate.

Method Validation

System Suitability:

The rationale of the system suitability assessment is to make sure that during the complete testing, system (including instrument, reagents, columns, analysts) is appropriate for the intended application.

System suitability tests (SST) are vital part of liquid chromatographic methods. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done. SST is support on the concept that the equipment, electronics, analytical operations and samples to be analyzed comprise an integral system that can be evaluated as such. The system suitability test was performed in accordance with USP [18].

Specificity:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or expected to present. The specificity of the developed LC method for all impurities was carried out by injecting placebo, known impurities of UDCA. The placebo was prepared by dissolving 10mg placebo in 10 ml of volumetric flask in mobile phase and sonicated. The volume was made up to the mark with diluents. The resulting solution was filtered through 0.45µ syringe filter. Each impurity was injected at nominal concentration (limit level). Preparation was done as per standard preparation.

The diluents (blank), placebo solution, individual impurities and standard drug solution (100µg/ml) were injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

Forced Degradation:

The forced degradation studies in acidic, hydrolysis, Oxidation and thermal condition were carried out during development which confirmed that UDCA is sensitive to oxidative conditions, potential degradation products forms at this condition. This study also confirmed that there is no co-elution of blank (Fig. 2), placebo (Fig. 3) or other substance with the principal peak (Fig. 4 and Fig. 5).

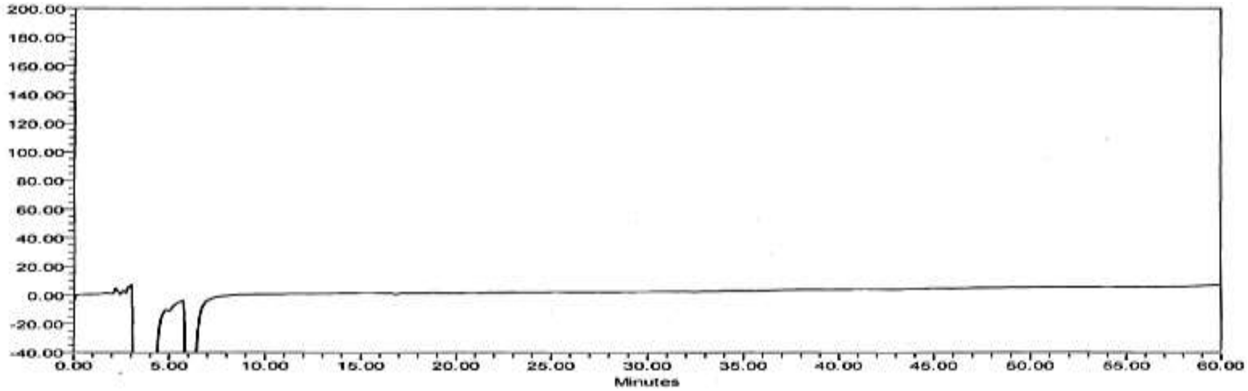

Figure 2 Blank Chromatogram

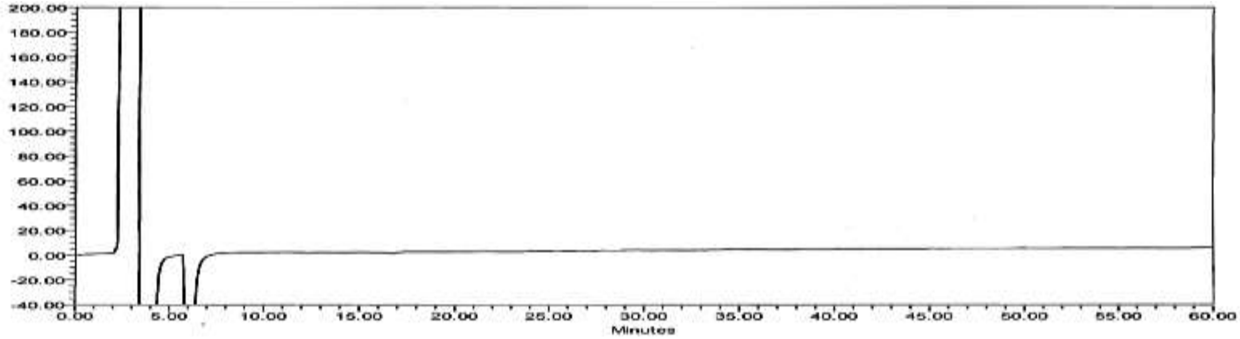

Figure 3 Placebo Chromatogram

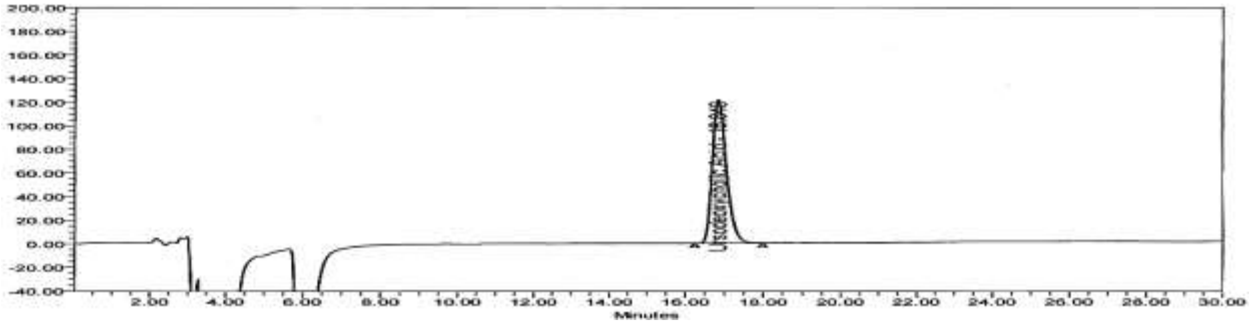

Figure 4 Standard Chromatogram

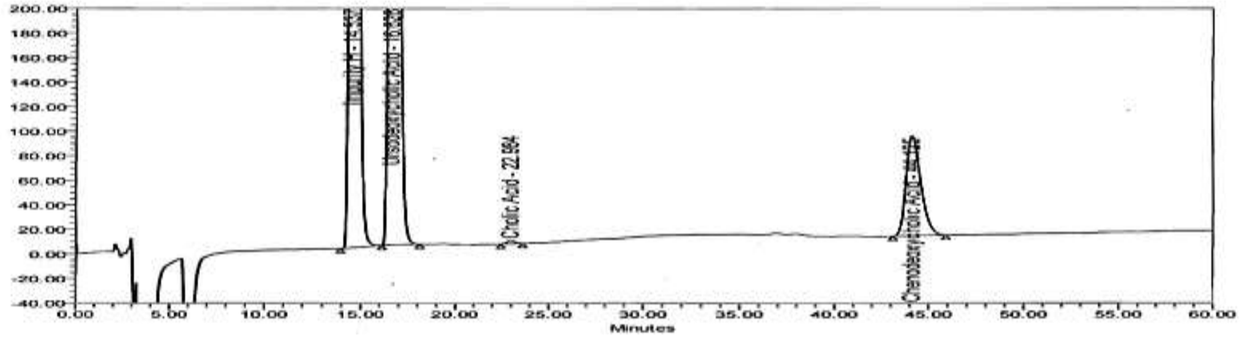

Figure 5 Chromatogram of Impurity and Sample

Precision:

Precision express the measure of how close the analytical results are to each other from a set of measurements under controlled analytical conditions. Precision proves random errors of the measurement.

Precision is a measure of the degree of repeatability (Intra-day), intermediate precision and reproducibility (inter-day) of the analytical method under normal operating circumstances. Precision is usually measured as the coefficient of variation or relative standard deviation of analytical results acquired from independently prepared quality control standards.

Method precision was evaluated by six sample preparations of same homogeneous sample of UDCA tablet test sample and calculated % recovery for known impurities in each sample preparation. The %RSD for set of six preparations was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out six sample preparation of tablets and calculated % recovery for impurities in each preparation. Calculated the %RSD for 12 results. The acceptance criteria for % RSD is not more than 15%.

Recovery (Accuracy):

Accuracy is extremely important in analytical method validation as it assures the closeness of agreement between a test result and the accepted reference value. Accuracy is expressed as trueness and involves a combination of random components and a common systematic error or bias component. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed methods, a recovery test was performed by adding known amounts of standard solutions to the placebo formulation sample, followed by analysis using the proposed chromatographic method.

The recovery studies were done for three different levels at LOQ (20%), 100% and 150% with three determination of working level concentration using standard spiking method in placebo.

All the above solutions were prepared in triplicate and were analyzed using proposed chromatographic condition. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery was in the range of 90 - 110%.

Linearity:

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range. The linearity plot was constructed for known impurities in the concentration range of LOQ to 150% impurity limit level. The primary stock solution of UDCA working standard and impurity solution was prepared. From the primary stock solutions, appropriate dilutions were made to get concentration of LOQ, 50, 80, 100, 120 and 150%. The calibration curve was plotted as concentration of the respective drug and impurity solutions versus the peak area at each level. The results were statistically evaluated and correlation coefficient determination (r^2), slope and y-intercept values were calculated.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. One consequence of evaluation of robustness is that a series of system suitability parameters is established to ensure that the analytical procedure is maintained whenever used. In the present study the working concentration 9000.0µg/ml of UDCA was used for the determination of the robustness of the method. The following parameters were considered for the robustness of the proposed chromatographic method.

- Effect of flow rate (±10%)
- Column oven temperature (±5°C)

Solution stability of sample and standard in mobile phase:

The solution stability of sample solution in diluents was performed to understand stability which will be helpful to understand sample handling in proposed chromatographic method. Solution stability was carried out for sample solution (100.0µg/ml) in a tightly capped volumetric flask at ambient temperature for 72 hr. The sample and standard solution after preparation were injected immediately to the system considering as an initial at 0 hr as baseline.

RESULTS AND DISCUSSION

Method Validation

Specificity:

No peak(s) due to blank and placebo were observed at the retention time of ursodeoxycholic acid, chenodeoxycholic acid (impurity A) and impurity H and cholic acid.

The RT and RRT data of known individual related substances confirmed that all known individual related substances are well separated.

The chromatogram of diluent, placebo, known impurities and sample solution were reveled that there is no co-elution of any impurity with drug peak. Based on overall outcome of degradation studies, it is concluded that method is specific.

Forced Degradation:

Forced degradation was done in different stressed conditions like acidic (0.5M HCl), basic (0.1N NaOH), oxidation (30% Hydrogen Peroxide) and thermal (was directly exposed to heat at 80°C for

48 hours) and the peak purity of UDCA was monitored. Peak purity of UDCA and impurity peak was found passing in all conditions (Table 1). The developed chromatographic method was found to be highly specific for determination of impurities in UDCA tablets.

Based on overall outcome of degradation studies it is concluded that proposed chromatographic method is specific and stability indicating.

Table 1: Accuracy results of chenodeoxycholic acid with respect to Ursodeoxycholic acid

Accuracy Level	% Recovery	Mean % recovery	% RSD
LOQ	99.1	97.2	6.8
	102.7		
	89.9		
50%	98.1	100.2	2.2
	99.9		
	102.5		
100%	99.9	103.0	3.2
	106.5		
	102.5		
150%	102.6	101.8	0.7
	101.5		
	101.4		
Overall Mean		100.6	4.0

Precision:

The intra-day precision was evaluated by performing analysis six (n = 6) times on same homogeneous sample of UDCA tablets and the % RSD was found to be 1.6%.

The % RSD for inter day precision for two sets (n=12) for their % recovery was found to be 2.2%.

Accuracy (Recovery):

The % recovery at LOQ, 50%, 100% and 150% of chenodeoxycholic acid was found to be 97.2±6.8, 100.2±2.2, 103.0± 3.2, and 101.8±0.7 % respectively (Table 1). The overall mean recovery was found to be 100.6± 4%. The recovery results were found within acceptance criteria (Table 1). The developed method found to be accurate for determination of impurities in UDCA tablets.

Linearity:

The linearity of the response of UDCA drug and chenodeoxycholic acid [impurity A] was performed in the range of LOQ-150% of impurity specification level and response for both found to be linear. The graphs were plotted for UDCA and chenodeoxycholic acid [impurity A] related substances with concentration on X-axis and area response on Y-axis. Correlation coefficient (C.C) ‘r’ and % Y intercept were calculated. The representative regression equation for UDCA was found to be y = 1.351x - 0.055 and correlation coefficient (r^2) was found to be 0.999. The representative regression equation for chenodeoxycholic acid [impurity A] was found to be y = 1.306x - 0.498 and correlation coefficient (r^2) was found to be 1.000. The linearity was found with in acceptance criteria.

Limit of detection [LOD] and Limit of quantitation [LOQ]:

The limit of detection and limit of quantification for chenodeoxycholic acid (impurity A) and UDCA was determined by signal to noise ratio method determined from linearity study (refer section 6). % LOD and LOQ values were reported w.r.t nominal sample concentration of UDCA[9mg/mL].

The results of LOD and LOQ are tabulated in Table 2.

Table 2: Summary of LOD and LOQ values of analyte by S/N ratio

Analyte	**Concentration [µg/mL]**		**Signal to Noise ratio**		**% w.r.t UDCA [9mg/mL]**	
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**
Ursodeoxycholic acid	1.4842	4.9473	4	15	0.016%	0.055%
Chenodeoxycholic acid	5.5979	13.9948	6	14	0.062%	0.155%

Robustness:

The robustness of the method was verified by making the deliberate variation in the critical method parameters like;

- Pump flow rate - ±10%)
- Column oven temperature - ±5°C

The results are presented in below table and found within acceptance criteria.

Solution stability of sample and standard:

The UDCA sample found to be not stable more than 2hour in diluents at ambient temperature. Based on the data, it is recommended and concluded that the standard solution can be used up to 5 days from the date of preparation when stored at room temperature [25°C] or at 2-8°C.

Table 3: Impact of change in flow rate of mobile phase on related substances data

% Impurity	**Flow rate 0.8mL/min [Normal conditions]**		**Flow rate 0.7mL/min**		**Flow rate 0.9mL/min**	
	RRT	**%Imp**	**RRT**	**%Imp**	**RRT**	**%Imp**
Chenodeoxycholic acid (impurity A)	2.870	1.525	2.801	1.605	2.818	1.549
Any other impurity (impurity H)	0.901	0.073	0.909	0.081	0.907	0.074
Sum of total impurities (including chenodeoxycholic acid)	-	1.598	-	1.686	-	1.623

Table 4: Impact of change in column oven temperature on related substances

% Impurity	**Column oven temperature 40°C [Normal conditions]**		**Column oven temperature 35°C**		**Column oven temperature 45°C**	
	RRT	**%Imp**	**RRT**	**%Imp**	**RRT**	**%Imp**
Chenodeoxycholic acid (impurity A)	2.870	1.525	3.009	1.523	2.752	1.616
Any other impurity (impurity H)	0.901	0.073	0.894	0.079	0.907	0.079
Sum of total impurities (including chenodeoxycholic acid)	-	1.598	-	1.602	-	1.695

Application of developed method:

The proposed chromatographic method was used for determination of impurities in UDCA tablets and the results were found within the specification.

CONCLUSION:

The proposed HPLC method is simple, precise, accurate and stability indicating rugged methodology for determination of impurities in UDCA tablets of different strengths. The validation of the method proven that the method is linear in the range of LOQ –150 % of impurity concentration limit, and it is proved to be precise and accurate over the range. The validation of the method is done accordingly to ICH Q2 (R1) and USP 38. All the parameters are meeting the acceptance criteria. Since method is simple and stability indicating hence it can be used conveniently by laboratories to determine the impurities of different formulations of Ursodeoxycholic Acid.

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