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Development and Validation of a Stability-Indicating Method for the Estimation of Ursodeoxycholic Acid using the RP-HPLC method

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ABSTRACT

Given that currently published analytical methods of ursodeoxycholic acid (UDCA) involve complex procedures and equipment, the purpose of this paper was to present the validation of a simple, rapid, accurate and precise isocratic UV and stability indicating RP-HPLC method for quantification residual solvents of ursodeoxycholic acid. Such a method would be critical in prospective developers. Validated method demonstrated Ursodeoxycholic acid separation without interference from the solvents with the most relevance for developing of tablet pharmaceutical drug carries. Method was linear over studied Ursodeoxycholic acid concentration range (2-10 µg/mL) with acceptable precision and accuracy. Method validation is carried out by using linearity (regression coefficient =0.934), accuracy (96.08 to 98.25), precision (intraday= % R.S.D. – 0.98and interday = % R.S.D. - 1.52), LOD (0.1) and LOQ (0.303), robustness (change in flow rate, change in wavelength and mobile phase composition was found to be 3.383 ± 3.47 respectively). Development and validation of Ursodeoxycholic acid was successfully carried out by using UV and HPLC method.

Keywords : RP-HPLC, Stability indicating, Ursodeoxycholic acid, Validation

I. INTRODUCTION

Ursodeoxycholic acid (ursodiol) is a naturally occurring bile acid found in the bile of the Chinese black bear. Black bear bile has been used for many years by practitioners of Eastern medicine and has been commercially synthesized and available for use as a hepatoprotective agent in Japan since the 1930s. Since the 1970s ursodeoxycholic acid has been used in Western human medicine for dissolution of gallstones. Ursodeoxycholic acid is an epimer of [chenodeoxycholic acid]. It is a mammalian bile acid found first in the bear and is apparently either a precursor or a product of chenodeoxycholate. Its administration changes the composition of bile and may dissolve gallstones. It is used as a cholagogue and choleretic

A literature survey revealed that several analytical tec hniques have been identified for testing ursodeoxycho lic acid as single drugs.However, very few methods ha ve been identified for estimating the ursodeoxycholic acid in hepatoprotective in biological samples as singl e drug candidates in biological fluids.The literature su

rvey revealed studies on analytical methods such as U V VIS, HPLC, LC MS, LC MS MS, and HPTLC for the determination of ursodeoxycholic acid Moreover, the methods described are not very costeffective in terms of solvent usage and overall runtime of analysis; henc e the present study was conduct.

The exhaustive literature survey revealed that none of the most recognized pharmacopoeias and journals include these drugs in **single** determination of Ursodeoxycholic acid, and information regarding the stability of the drugs is not available. Therefore, it seemed essential to develop a liquid chromatographic procedure that serves as a reliable, accurate and stability-indicating HPLC method for the simultaneous estimation of Ursodeoxycholic acid in tablet dosage form. The present investigation was conducted with the goal of establishing a simple, rapid, and robust stability-indicating RP-HPLC method for the simultaneous estimation of Ursodeoxycholic acid in bulk and in tablet form.

II. EXPERIMENTAL

2.1 Chemicals and reagents

All reagents and solvent were of analytical grade Abbout pharma PVT.Ltd kindly provided bulk formulation and other all chemical substance of analytical lobachem PVT. Ltd reagent grade unless otherwise specified all solution have been formulated using methanol and acetonitrile. High- purity deionized water was obtained using and all the other chemicals used were of analytical grade. Doubly distilled water was used for preparing the mobile phase solutions.

2.2 Instrumentation

A JASCO-HPLC 2075 plus isocratic high pressure liquid chromatograph (Japan) with an LC-P-2089 pump, an LC-UV 2075 variable wavelength programmable UV/Vis detector were used. Chromatographic separation was performed by a reverse-phase Thermofisher Pak C_{18} column (4.6 mm × 250 mm with a particle size of 5 μm).

2.3 Chromatographic conditions

The mobile phase, which consisted methanol: acetonitrile: (48:52 v/v), was degassed and filtered using a Millipore vacuum filter system equipped with a 0.45 μm membrane filter. Chromatography was performed at an ambient temperature by pumping the mobile phase at a flow rate of 1.0 ml/ min^{-1}. The column's effluence was monitored at 216 nm.

2.4 Preparation of the stock solution

Accurately weighed of ursodeoxycholic acid (10 mg) were transferred to a 10.0 ml volumetric flask. Then, a small amount of the mobile phase was added and the result was ultrasonicated for 15 min and diluted up to the mark with the mobile phase to obtain a final concentration of 1000 μg mL^{-1}.

2.5 Preparation of the standard working solution

From the Ursodeoxycholic acid stock solutions, 1 ml was transferred to a 10 ml volumetric flask by pipetting, and the final volume was made up with the mobile phase to obtain a final concentration of 100 μg mL^{-1}.

2.6 Preparation of the working sample solution

The powders from twenty tablets containing 200 mg Ursodeoxycholic acid were weighed. A quantity of powder that was equivalent to 10 mg was placed in each of several 10 mL volumetric flasks that contained approximately 10 mL of the mobile phase for analysis, and the result was sonicated for 15 min. After sonication, the volume was made up to the mark using the same solution to obtain the sample stock solution of Ursodeoxycholic acid. Then, the solution was filtered using a 0.45 μm membrane filter. The filtrate (0.010 mL) **was** quantitatively transferred to a 10 mL

volumetric flask to obtain final concentrations of 100 μg mL^{-1} for Ursodeoxycholic acid.

2.7 Method validation

For quantitative determination it was found that the chromatographic conditions mentioned in the present manuscript are sufficient. After optimization of the analytical conditions, other parameters such as linearity, precision, accuracy (recovery), selectivity and robustness were evaluated to validate the process

2.7.1 System suitability test

System suitability checks in compliance with USP 24/NF 19 were conducted to ensure that the reproducibility of the system was sufficient for analysis [31]. The study was conducted to ensure reproducibility of the chromatographic method prior to the analysis of each batch of samples. The chosen parameters were predicated on the method's actual performance, as calculated during its validation. Such parameters include the relative standard deviation of the retention times (percent RSD), tailing component, theoretical plate and asymmetry for six injection repetitions.

2.7.2 Linearity

The linearity was studied using six concentrations, 2, 4, 6, 8, and 10μg mL^{-1}, of Ursodeoxycholic acid. The linearity analysis was carried out four times to ensure linear reaction of the detector for various concentrations of drugs (2–10 μg mL^{-1}). Working conditions were optimized by applying to the necessary range concentration of various concentrations; the solutions were then inserted into the HPLC device. The calibration curves were developed by plotting the peak region versus ursodeoxycholic acid concentrations, and the regression equations were calculated.

2.7.3 Accuracy (% recovery)

The HPLC method's precision and accuracy may also be improved and also corrects variations in the response of the detector. An analysis of the accuracy was carried out using the standard method of introduction. The prequantified sample solutions of ursodeoxycholic acid (2.00 μg mL^{-1}) were spiked with an extra 0, 60, 80, and 100% of the standard Ursodeoxycholic acid solutions, respectively. Those mixtures have been analyzed using the method developed. The procedure was carried out four times. For increasing concentration the percentage of sample recovery, percentage of RSD, and percentage is determined.

2.7.4 Precision

The intra-day and inter-day precision were estimated for three different concentrations (5, 10 and 15 μg mL^{-1}) of Ursodeoxycholic acid four times on each of three days to obtain the relative standard deviation (%RSD).

2.7.5 The LOD and the LOQ

The ICH Guidelines define various approaches for evaluating the identification (LOD) and quantification (LOQ) limits in this analysis, the LOD and the LOQ were focused on the standard response and slope variance using the signal-to-noise ratio according to the ICH guidelines

2.7.6 Robustness

The robustness of the established technique was calculated to determine the effect of the chromatographic conditions on a low so purposeful variance. The robustness of the method was measured by changing the flow rate (0.5 and 1 mL min^{-1}) of the mobile phase, the methanol: acetonitrile (48:52).

2.7.7 Selectivity

The selectivity was verified by comparing the chromatograms obtained for the standard, the sample and the corresponding placebo.

2.8 Forced degradation

The guidance of the International Conference on Harmonization (ICH) entitled "Stability Testing of Modern Drug Substances and Products" includes

stress testing to elucidate the inherent stability properties of an active substance. The aim of this research was to examine the stress degradation of ursodeoxycholic acid using the proposed method.

2.8.1 Hydrolytic degradation under acidic conditions

Using 10 µg ml stock solutions of Ursodeoxycholic acid, respectively, 5 mL of a stock solution and 1 mL of 0.1 N HCl were added to a 10 mL volumetric flask. Then, the volumetric flask was kept under 60–70 °C reflux conditions for 4 h and neutralized with 0.1 N NaOH and 10 mL of a diluent to obtain 10 µg ml concentrations of Ursodeoxycholic acid , respectively. The solution was cooled to room temperature, filtered using 0.45 µm syringe filters and placed in the vials of the HPLC system.

2.8.2 Hydrolytic degradation under alkaline conditions

Using 10 µg ml stock solutions of Ursodeoxycholic acid, respectively, 5 mL of a stock solution and 1 mL of 0.1 N NaOH were added to a 10 ml volumetric flask. Then, the volumetric flask was kept under 60–70 °C reflux conditions for 4 h and neutralized with 0.1 N HCl. Then, 10 mL of a diluent was added to obtain 10 µg mL **concentrations** of Ursodeoxycholic acid, respectively. The solution was cooled to room temperature, filtered using 0.45 µm syringe filters and placed in the vials of the HPLC system.

2.8.3 Oxidative degradation

Using 10 µg mL stock solutions of Ursodeoxycholic acid, respectively, 5 mL of a stock solution and 1 ml of 3% (w/v) of hydrogen peroxide were added to a 10 mL volumetric flask. The volumetric flask was then kept under 60–70 °C reflux conditions for 1 h, and the volume was made up to the mark with diluents to obtain final Ursodeoxycholic acid concentrations of 10 µg mL respectively. The solution was cooled to room temperature, filtered using 0.45 µm syringe filters and placed in the vials of the HPLC system.

2.8.4 Thermal induced degradation

Using 10 µg ml stock solutions of Ursodeoxycholic acid, respectively, 5 mL of a stock solution was added to a 10 ml volumetric flask. Then, the volumetric flask was kept at 60–70 °C in a hot air oven for 24 h, and the volume was made up to the mark with diluents to obtain 10 µg mL concentrations of Ursodeoxycholic acid , respectively. The solution was cooled to room temperature, filtered using 0.45 µm syringe filters and placed in the vials of the HPLC system.

2.8.5 Photo degradation

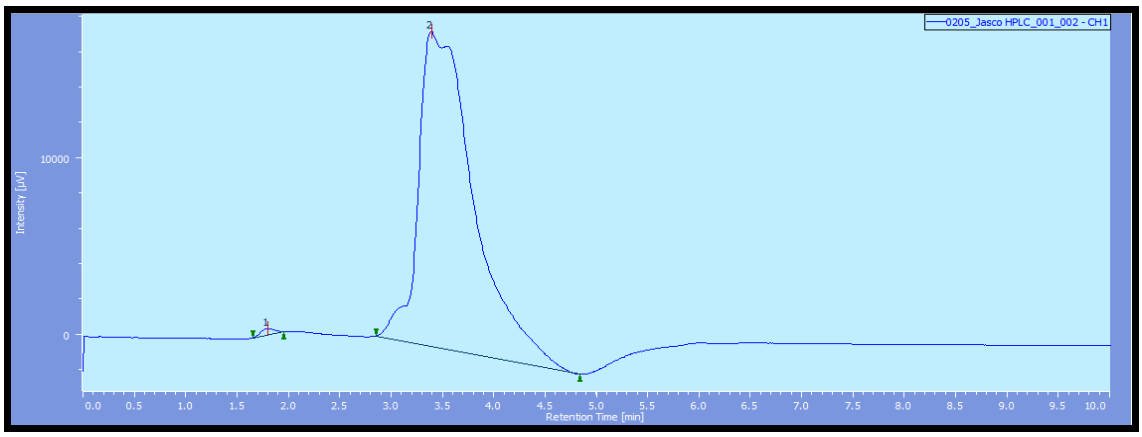
Using 10 µg ml stock solutions of Ursodeoxycholic acid, respectively, 5 ml of a stock solution was added to a 10 ml volumetric flask. The samples were transferred to petri dishes and kept in photo stability for 7 days. Then, the volumetric flask was made up to the mark with diluents to obtain concentrations of Ursodeoxycholic acid, respectively. The solution was cooled to room temperature, filtered using 0.45 µm syringe filters and placed in the vials of the HPLC system.

III. RESULTS AND DISCUSSION

Optimized chromatromatographic conditions:

To optimize the RP-HPLC parameters and reach a good resolution and a peak shape for ursodeoxycholic acid, several chromatographic conditions were tested. Several mobile phases of different compositions were tested to find one that provided sufficient selectivity for the drugs. The phosphate buffer provided a higher sensitivity and selectivity than other buffers did. Using methanol and acetonitrile as organic components resulted in higher sensitivity, but varying the amounts of

methanol and acetonitrile in the mobile phase affected the resolution and run time. Methanol:Acetonitrile (48:52v/v). The coumn effluence was monitored at 216 nm. The optimal injection volume was 10 µL. The column's temperature was maintained at 25 °C (ambient). The Chromatographic separation was performed by a reverse-phase thermofisher Pak C_{18} column (4.6 mm × 250 mm with a particle size of 5 µm). Was used in its isocratic mode with a flow rate of 1 mL min^{-1}. Retention times of approximately 3.375 and 3.408 min were consistently observed for Ursodeoxycholic acid, respectively, in all the analytical runs (Fig. 1).



#	Peak Name	CH	tR	Area	Height	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor	Warning
1	Peak-001	1	1.800	3488	362	0.515	1.997	N/A	728	2.745	1.026	Calibration Curve
2	ursodeoxycholic acid	1	3.392	674146	17792	99.485	98.003	N/A	229	N/A	1.805	Calibration Curve

Figure 1. Representative chromatogram of ursodeoxycholic acid

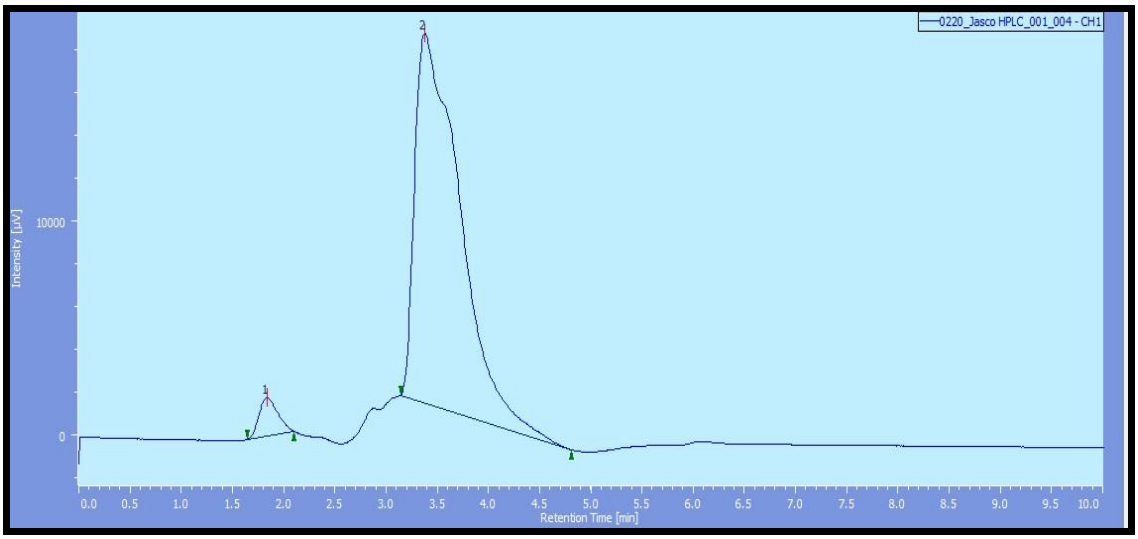
Forced degradations studies:

Summary of degradation product was tabulated in table No.1

1) **Table 1. Summary of degradation products of ofloxacin**

Condition	Time (in hrs)	Drug peak area at zero hrs	Drug peak area stressed sample	Retention time of degradant (mins)	% Degradation
1 N HCL	4hr	674146	531900	3.1,3.3	7%
1 N NaOH	4hr	677826	531477	3.5,3.3	12%
H2O2, 3%	1hr	665214	574837	3.2,3.3	8%
Hot air oven	24hr	658425	571254	3.6,3.4	11%
Photo stability	7 days	671542	669560	3.4,3.4	9%

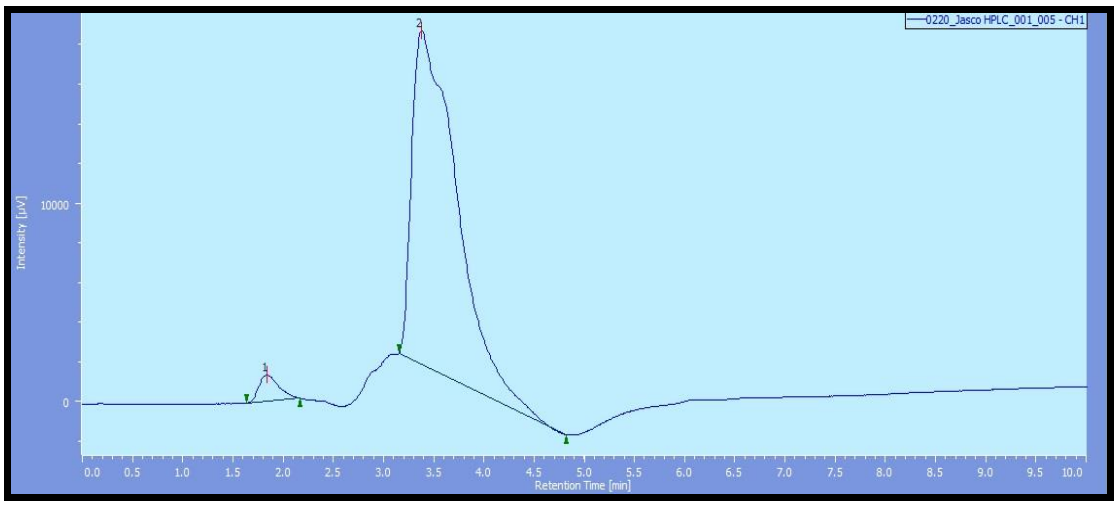
Acidic degradation: Ursodeoxycholic acid showed sufficient degradation at 4 hrs, in 1 N HCL for 60^0-70^0. The degradation product formed for ursodeoxycholic acid was at retention time (Rt) 3.3 min. It has shown in fig.2



#	Peak Name	CH	tR	Area	Height	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor	Warning
1	Peak-001	1	1.842	22504	1759	4.059	9.253	N/A	455	2.620	1.213	Calibration Curve
2	Unknown	1	3.375	531900	17249	95.941	90.747	N/A	266	N/A	2.779	

Figure 2. Hydrolytic degradation under acidic conditions of Ursodeoxycholic acid

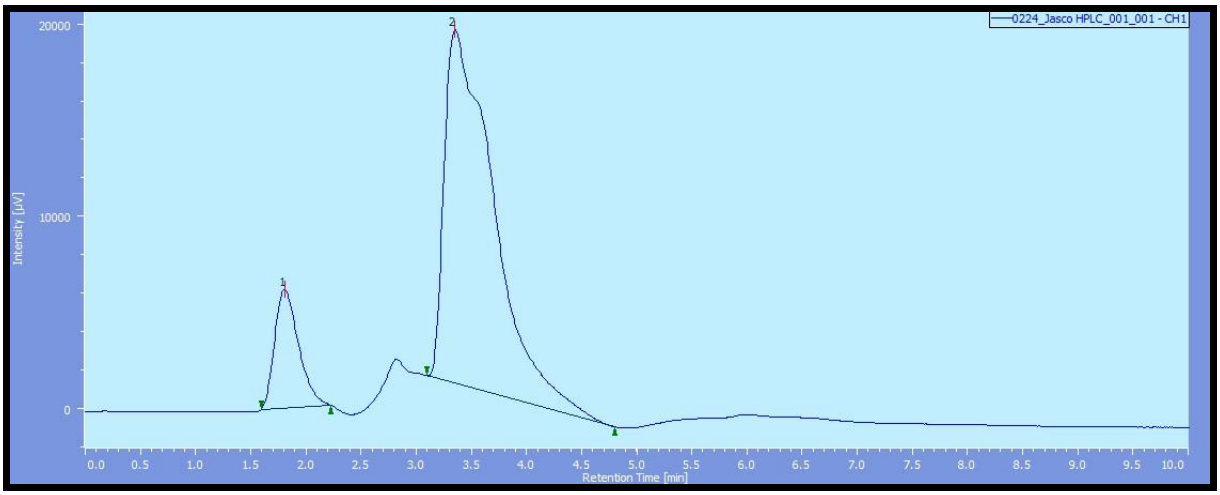
Alkali degradation: The drug shown sufficient degradation in alkaline hydrolysis in 1 N NaOH at 60^0-70^0 C. The degraded product appeared at Rt 3.3 min for ursodeoxycholic acid. It has shown in fig.3.



#	Peak Name	CH	tR	Area	Height	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor	Warning
1	Peak-001	1	1.833	18404	1328	3.347	7.325	N/A	407	2.585	1.439	Calibration Curve
2	Unknown	1	3.375	531477	16805	96.653	92.675	N/A	263	N/A	2.901	

Figure 3. Hydrolytic degradation under alkaline conditions of Ursodeoxycholic acid

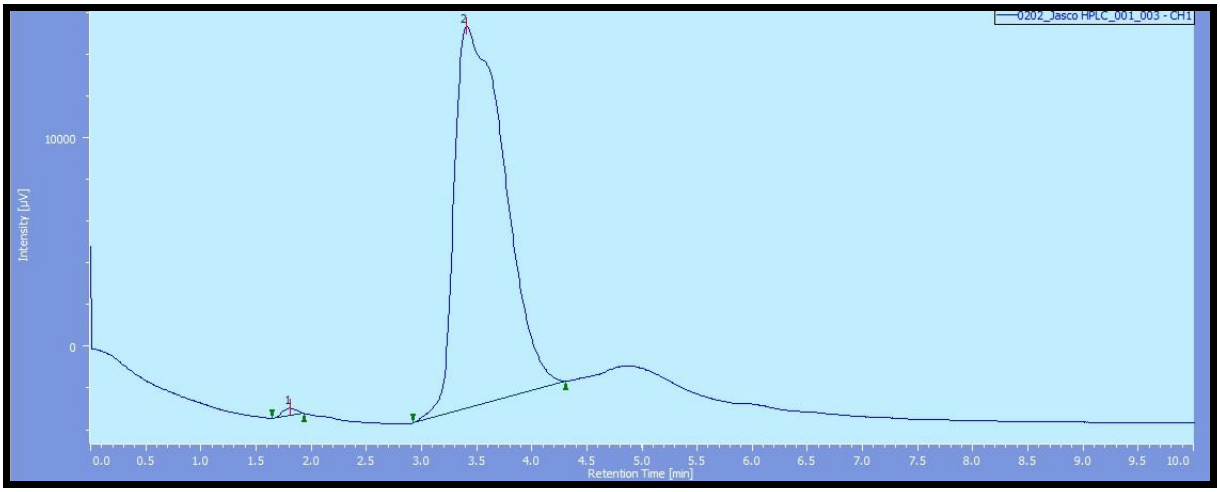
Oxidative degradation: The drug ursodeoxycholic acid sufficient degradation in 3 % H2O2 kept 1 hrs for 60^0-70^0 C temperature. The degraded product appeared at Rt 3.3 min ursodeoxycholic acid. It has shown in fig.4



#	Peak Name	CH	tR	Area	Height	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor	Warning
1	Peak-001	1	1.808	93283	6148	13.962	25.071	N/A	328	2.527	1.376	Calibration Curve
2	Unknown	1	3.350	574837	18375	86.038	74.929	N/A	265	N/A	3.042	

Figure 4. Oxidative degradation conditions of Ursodeoxycholic acid

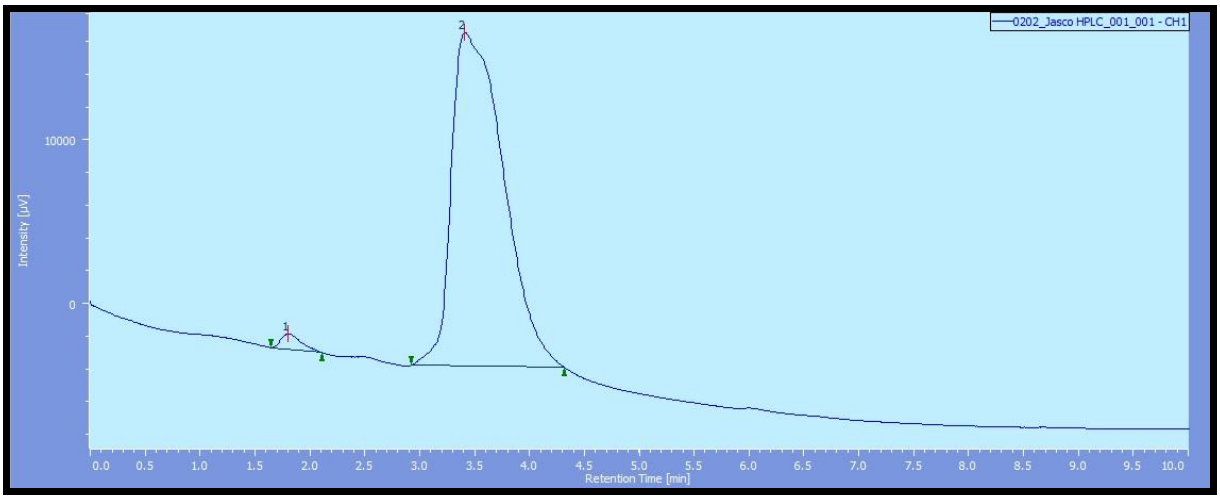
Thermal degradation: The thermal degradation product was in oven at 60^0- 70^0 C for 24 hrs at Rt 3.4 min. It has shown in fig.5.



#	Peak Name	CH	tR	Area	Height	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor	Warning
1	Unknown	1	1.808	3089	348	0.538	1.869	N/A	809	2.872	0.970	
2	Unknown	1	3.408	571254	18262	99.462	98.131	N/A	250	N/A	1.972	

Figure 5. Thermal induced degradation of Ursodeoxycholic acid

Photo degradation: The photo degradation product was in petri dishes and kept in photo stability for 7 days at Rt 3.4 min. It has shown in fig.6.



#	Peak Name	CH	tR	Area	Height	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor	Warning
1	Unknown	1	1.800	12062	943	1.770	4.437	N/A	462	2.639	1.554	
2	Unknown	1	3.408	669560	20311	98.230	95.563	N/A	236	N/A	1.749	

Figure 6. Photo degradation of Ursodeoxycholic acid

3.1 Validation of the proposed method

The developed stability indicating method was validated according to ICH guidelines. The validation parameters addressed were linearity, precision (inter-day, intra-day and intermediate precision), accuracy and LOD, LOQ, robustness.

Linearity: From the Linearity data it was observed that the method was showing linearity in the concentration range of 2-10 µg/ml at 216 nm for ursodeoxycholic acid. Correlation coefficient (R^2) was found to be 0.943 for the compound. The linearity data was tabulated in Table.2. The Chromatograms for the linearity data were shown in the fig no: and the linearity curve was plotted and given in the Fig.7

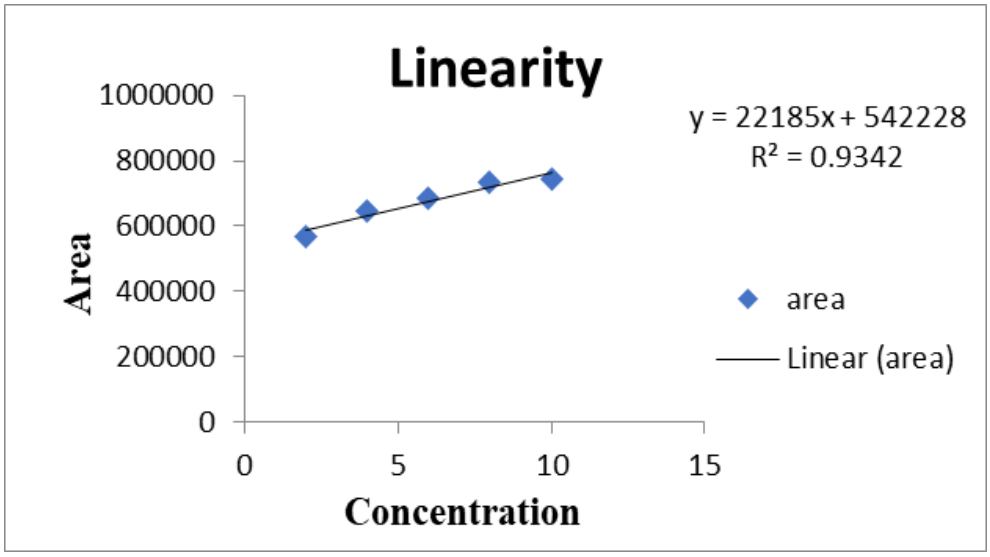


Figure 7. Calibration curve of ursodeoxycholic acid

Table 2. Linearity data for ursodeoxycholic acid

Calibration standard	Nominal Concentration µg/ml	Peak Area of replicate (µV.sec)			Mean peak area	Standard deviation of peak area	% RSD
		1	2	3			
1	2	566463	564585	567814	566287	1621.652	0.286366
2	4	642763	647871	648754	646462	3234.281	0.500304
3	6	688299	684542	682478	685106	2951.248	0.430772
4	8	736281	732012	736541	734944	2543.089	0.346025
5	10	744326	745815	741558	743899	2160.285	0.290445
Equation	$Y = 22185X + 54222$ $r^2 = 0.934$						

Specificity: The Chromatograms of Standard and Sample are identical with nearly same Retention time. There is no interference with blank and placebo to the drugs. The chromatograms were shown in the Figures 1 for standard, sample, blank and placebo.

Accuracy: Recovery studies of the drugs were carried out for determining accuracy parameter. It was done by mixing known quantity of standard drugs with the pre-analyzed sample formulation and the contents were reanalyzed by the proposed method. This was carried out at 60, 80 and 100% levels. The percentage recovery and its % RSD were calculated. The method was found to be accurate with percent recoveries between 96.8 and 98.25 and % RSD was < 2. The results were tabulated in the Table.3.

Table 3. Accuracy data of ursodexycholic acid

% level of recovery	Initial Amount	Amount Added	Area	Amount found	% Amount found	Amount Recovery	% Recovery
60 %	4	3.2	688299	6.1452	96.5845	4.1452	96.5845
	4	3.2	684542	6.1485	96.4572	4.1485	96.4572
	4	3.2	682478	6.1245	96.5241	4.1245	96.5241
			Mean	6.1394	96.5219	4.1394	96.5219
			SD	0.0139	0.0636	0.0130	0.0636
			%RSD	0.0130	0.0659	0.3142	0.0659
80%	4	4	736281	7.4514	92.5845	3.7248	92.9369
	4	4	732012	7.4174	92.4875	3.7595	92.9858
	4	4	736541	7.1267	93.9685	3.7584	93.0360
			Mean	7.3318	93.0135	93.0135	92.9862
			SD	0.1784	0.8284	0.0197	0.8284
			%RSD	0.2434	0.8907	0.5263	0.8907
100%	4	4.8	744326	8.4571	91.5345	4.8574	91.6604
	4	4.8	745815	8.7458	91.7895	4.6958	91.7010
	4	4.8	741558	8.3506	91.8574	4.2015	91.7220
			Mean	8.5178	91.7271	4.5849	91.7271
			SD	0.2044	0.1702	0.3417	0.1702
			%RSD	0.2400	0.1855	0.7453	0.1855

Precision: The %RSD for the sample chromatograms of method precision were found to be Intra- day 0.98 .& Intraday 1.52 for ursodeoxycholic acid . Hence it passes method precision. The results were tabulated in the Table.4

Ruggedness: Comparison of both the results obtained for two different Analysts shows that the method was rugged for Analyst-Analyst variability. The system suitability parameters of Ruggedness were found to be within the limits and were tabulated in Table.5. The Chromatograms for ruggedness were shown in Figures 1.

Table 4. Ruggedness data of ursodeoxycholic acid

	Sr. No.	Concentration	Area	Amount found	% Amt. found
Analyst 1	1	6	688299	5.97134411	99.522445
	2	6	684512	5.98016877	99.6694798
	3	6	687854	5.97980948	99.6634945
Analyst 2	4	6	685485	5.98006208	99.6677015
	5	6	689547	5.98000384	99.6667318
	6	6	682515	5.98015903	99.6693180
			Average	5.97859127	99.6432881
			SD		0.05919541
			% RSD		0.05940745

Robustness: There was no significant change in the retention time of ursodeoxycholic acid and its degradation products after introducing small changes in mobile phase composition indicating robustness of the method.

Table 5. Summary of Validated Parameters of ursodeoxycholic acid

Parameters	Ursodeoxycholic acid
Calibration range(µg/ml)	2-10 µg/ml
Correlation coefficient(R^2)	0.934
Precision(Intra-day)%RSD	0.9858
Precision(Inter-day)%RSD	1.52
Retention time	3.4
LOD	0.01
LOQ	0.303
Assay	96.08%

IV. CONCLUSION

The proposed RP-HPLC method is accurate, precise, rapid, robust, sensitive and selective. The prescribed method adapted the use of an economical and easily available mobile phase, a UV detector, and easy extraction procedures. Washing the column with the same mobile phase made it an excellent method for the quantification of Ursodeoxycholic acid in bulk drugs and in their pharmaceutical dosage forms. A stability-indicating RP- HPLC method for the estimation of Ursodeoxycholic acid in their solid dosage forms was established and validated in accordance with the ICH guidelines. The forced degradation experiment and the peak purity data confirmed that there was no merging of the peaks of the active ingredients with those of any other degradation products or other additives. The developed method can be used in routine analyses of drugs in bulk and in different formulations and could help in therapeutic drug monitoring (TDM) and stability studies.

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