A validated RP-HPLC method for quantitative determination of genotoxic impurity hydrazine content in Ursodeoxycholic acid

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Abstract

A highly sensitive method for the determination of genotoxic impurity such as hydrazine in Ursodeoxycholic acid using RP-HPLC has been presented in the present study. Quantification of hydrazine content in Ursodeoxycholic acid sample by HPLC was done with UV Detector. Hydrazine was UV inactive compound. Derivatization procedure was established to detect the hydrazine in HPLC. For this, 4-Hydroxy 3-methoxybenzaldehyde was used as a derivatizing agent which reacts with hydrazine in the presence of disodium tetraborate to form a compound which was UV active. Hydrazine was determined by RP-HPLLC method using LiChrospher 100-RP18 (250 x 4.6mm, 5 µm) column as stationary phase.

Column temperature maintained 35°C, injection volume 100µL, flow rate was 1.0 mL/min. Hydrazine was detected using UV detector at the wavelength of 350 nm and run time was 45 minutes. The mobile phase used water and methanol in gradient mode. The method validation has been carried as per International Conference on Harmonization guidelines (ICH). Limit of quantitation (LOQ) was found 0.21 ppm for hydrazine.

Keywords: Genotoxic impurity, Hydrazine, Ursodeoxycholic acid, 4-Hydroxy 3-methoxybenzaldehyde, RP-HPLC method, Validation.

Introduction

Synthesis of drug substances often involves the use of reactive reagents and hence, these reagents may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities, including genotoxicity and carcinogenicity and are to be controlled based on the maximum daily dose[1]. These limits generally fall at low µg/mL levels. HPLC-GC methods (or final drug substance methods) are suitable for their determination. Their applications are oriented towards the potential identification and quantitation of trace level of impurities in drug substances[2].

The chemical name of Ursodeoxycholic acid is (3-alpha,5-beta,7-beta)-3,7- dihydroxycholan-24-oic acid and it is a naturally occurring bile acid found in humans in minute quantity[3,4]. It prevents billiary secretion of cholesterol and also diminishes cholesterol intestinal absorption[5,6]. It is used to dissolve cholesterol rich gallstones in patients with functioning gallbladders[7,8] and also in treatment of billiary cirrhosis[9], viral hepatitis[10,11], cystic fibrsosis etc.[12] Corresponding to the molecular formula $C_{24}H_{40}O_4$, it has a relative molecular mass of 392.56 g/mol.

Ursodeoxycholic acid is a white to off-white, not hygroscopic crystalline powder. It is freely soluble in ethanol, glacial acetic acid, slightly soluble in chloroform, sparingly soluble in ether, practically insoluble in water (20mg/L). Its pKa has been found to be 5.10 and its partition coefficient was found to be 3.0. Ursodeoxycholic acid is known to exhibit polymorphism. 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.

In the manufacturing process of ursodeoxycholic acid, hydrazine is used as reagent and hence genotoxic hydrazine may exist as impurity in ursodeoxycholic acid drug substance.

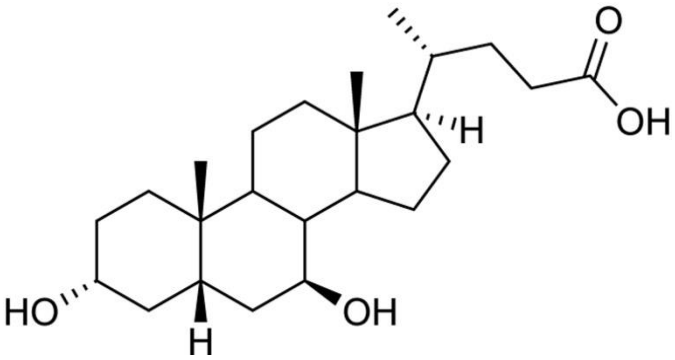


Figure 1: Chemical structure of Ursodeoxycholic acid

Impurity structure:

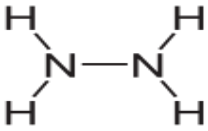
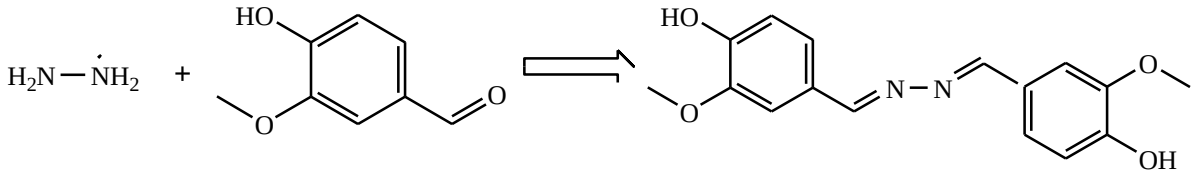


Figure 2: Chemical structure of Hydrazine (HDZ)

Hydrazine was UV inactive compound. Derivatization procedure was established to detect the hydrazine in HPLC. For this 4-Hydroxy 3-methoxybenzaldehyde was used as a derivatizing agent which reacts with hydrazine in the presence of disodium tetraborate to form a UV active compound.



Hydrazine 4-Hydroxy 3-methoxybenzaldehyde UV Active compound

The literature survey reveals that a few methods were used to detect and quantify hydrazine CAD[13-16], most of which use a derivatization approach coupled with analytical techniques ranging from GC[17-19], GC–MS[20], LC[21-23], LC–MS/MS[24-26], Capillary electrophoresis[27], electrochemical sensing[28-32] and ion chromatography (IC)[33].

In literature, no analytical method was reported for the determination of hydrazine in ursodeoxycholic acid. Hence the present study aimed towards the development of rapid, specific and robust methods for the determination of hydrazine in ursodeoxycholic acid at trace level concentration according to ICH guidelines[34].

Material and Methods

Materials: Hydrazine (HDZ) and 4-Hydroxy 3-methoxybenzaldehyde were purchased from Sigma-Aldrich., Mumbai, India. Methanol and disodium tetra borate were procured from Merck, India. Milli-Q water HPLC grade was procured from Merck, India.

Instrumentation: Waters HPLC model - e2695 with DAD, Bandelin ultrasonic bath, pH Meter (Thermo Orion Model) and Analytical Balance (Metller Toledo Model) were used in the present study.

Preparation of Mobile phase: Mix accurately water and methanol in the ratio of 60:40 (%v/v) and elute via gradient mode.

Preparation of Hydrazine stock solution: Weigh accurately 3.25 mg of hydrazine sulfate (equivalent to 0.8 mg of Hydrazine) into a 100 mL volumetric flask, add 10 mL of water sonicated to dissolve. Mix well and make up to the mark with methanol.

Preparation of Hydrazine standard solution (0.8 µg/mL): Transfer 1.0 mL of HDZ stock solution into a 10mL volumetric flask containing about 5mL of diluent. Mix well and make up to the mark with diluent. This solution is equivalent to 0.8 ppm of HDZ with respect to 100.0 mg/mL of sample solution.

Preparation of sensitivity solution (0.21 µg/mL): Transfer 5 mL of standard solution into a 20 mL volumetric flask, dilute to volume with diluent and mix well.

Preparation of sample solution: Weigh 1000.45 mg of the ursodeoxycholic acid into a 10 mL volumetric flask. Dissolved in 5mL of diluent and mix well, then make up to the mark with diluent.

Preparation of sample spiked solution: Weigh 1000.23 mg of the ursodeoxycholic acid into a 10mL volumetric flask. Dissolved in 5mL of diluent and added 1.0 mL of HDZ stock solution. Mix well and then make up to the mark with diluent.

Method development: To develop a suitable and robust HPLC method for the determination of hydrazine impurity in ursodeoxycholic acid drug substance, different column stationary phases were employed to achieve an efficiently separation of hydrazine impurity from blank, placebo and ursodeoxycholic acid analyte peak. The method development started with Waters X-bridge Shield RP-18 (250 x 4.6 mm, 5µ) with the following mobile phase compositions like mobile phase-A water and mobile phase-B acetonitrile in gradient mode. There was no proper separation of impurity from blank and placebo solution and peak interferences are present.

For the next trial except mobile phase B, the remaining parameters: column, flow rate, injection volume, wavelength are same. The mobile phase-B was changed from acetonitrile to methanol in gradient mode respectively. Flow rate 1.0 mL/min, column temperature 35°C and sampler cooler were maintained at 25°C. UV detection was performed at 353nm. There was no proper separation between hydrazine impurity peak and blank peaks and placebo peaks. For the next attempt, the column was changed from Waters X-bridge Shield RP-18 (250 x 4.6 mm, 5µ) to LiChrospher 100-RP18 (250 x 4.6mm, 5µm). The separation between hydrazine impurity peak and blank peaks and placebo peaks was achieved. These chromatographic conditions were selected for validation studies.

Optimised Chromatographic conditions: The chromatographic analysis was performed on Waters 2695 HPLC system. The chromatograms were recorded and analyzed by Empower[3] software. The separation was performed on LiChrospher 100-RP18 (250 x 4.6mm, 5µm), mobile phase consisting of mobile phase-A as water and mobile phase-B as methanol in gradient mode. The HPLC gradient program was time (min)/B% v/v: 0/40, 15/72, 23/72, 28/80, 35/80, 38/40, 45/40. The flow rate was 1.0 mL/min, the column oven temperature was 35°C and the sampler cooler temperature was 25°C. The injection volume was 100µL *and* detection was performed at 353 nm using a photodiode array detector (PDA).

Results

The developed method was validated as per ICH guidelines[5] in terms of Specificity, System suitability, Precision, Limit of Detection (LOD), Limit of Quantization (LOQ), Linearity and Accuracy.

Specificity (Interference from blank, placebo): Specificity was demonstrated by injected blank solution, placebo solution, standard solution, sample solution and spiked sample solution analyzed as per the optimized method. The observations are tabulated in table 1 and figure 3 to figure 7. Table 1 and figures 3 to 7 illustrate that the chromatograms were recorded for blank, placebo, standard, sample and spiked sample solutions of hydrazine impurity and ursodeoxycholic acid. Specificity studies reveal that the peaks are well separated from each other. Therefore, the method is selective for the determination of hydrazine impurity in ursodeoxycholic acid.

System suitability (System precision): Prepare a standard solution as per the test method and inject it six times into the HPLC system. The retention time and area of the analyte peak were recorded. The observations are tabulated in table 2.

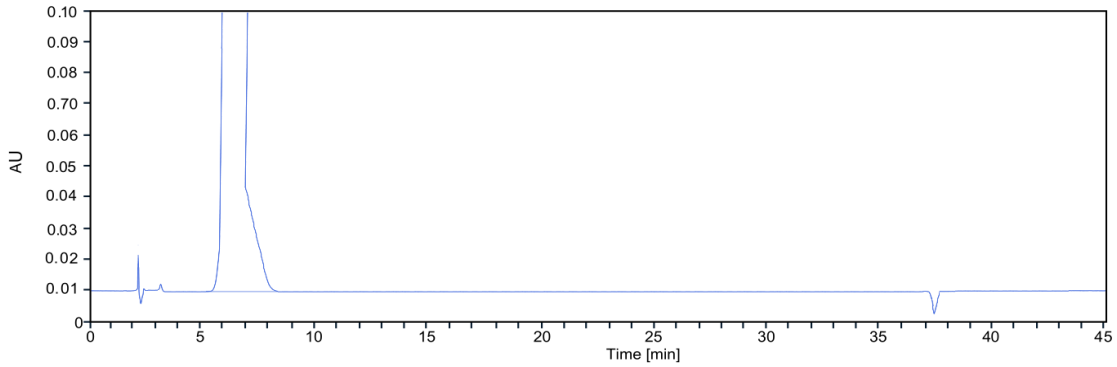


Figure 3: Typical chromatogram of Blank

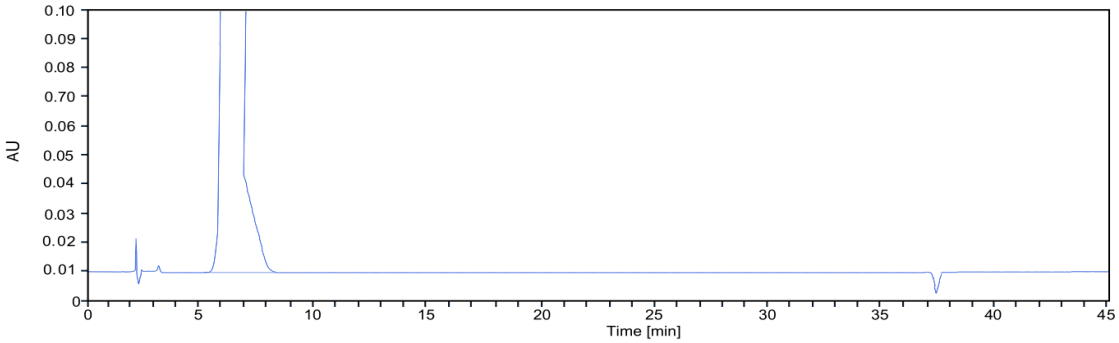


Figure 4: Typical chromatogram of Placebo

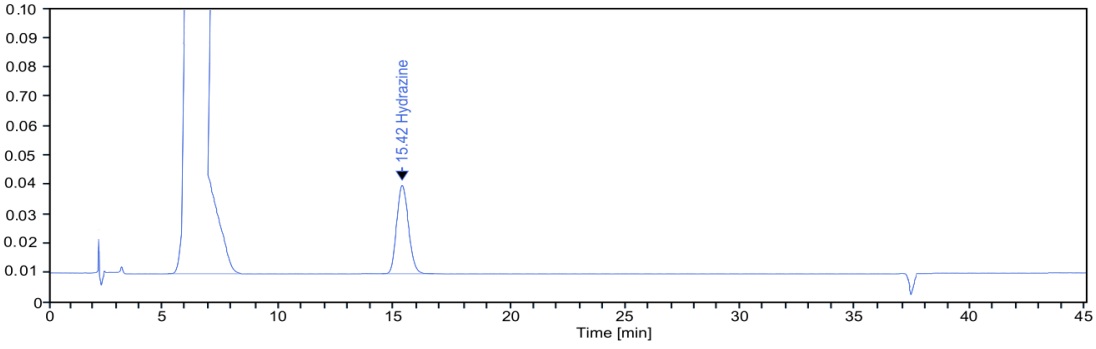


Figure 5: Typical chromatogram of Standard

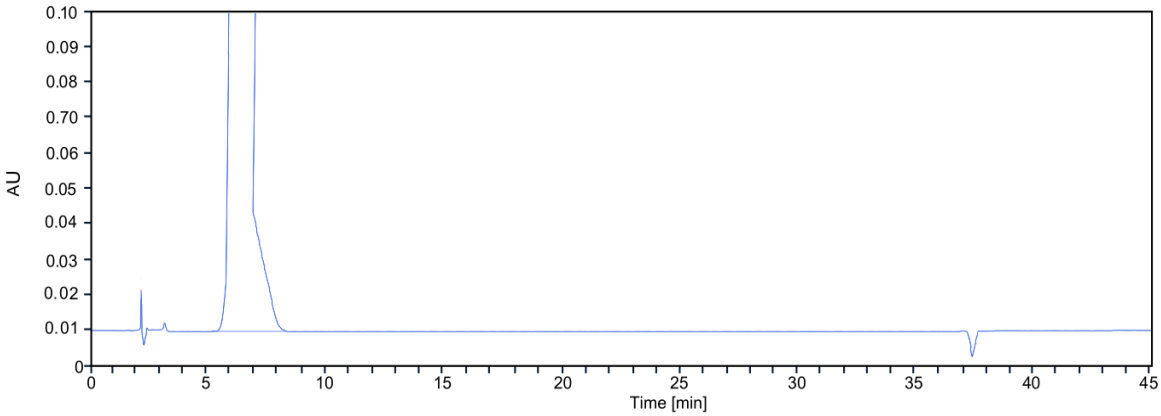


Figure 6: Typical chromatogram of Sample

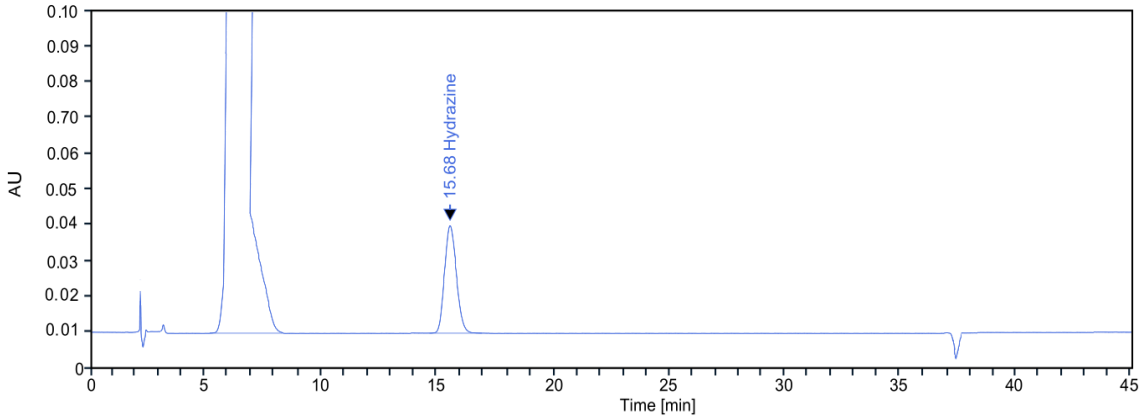


Figure 7: Typical chromatogram of spiked sample

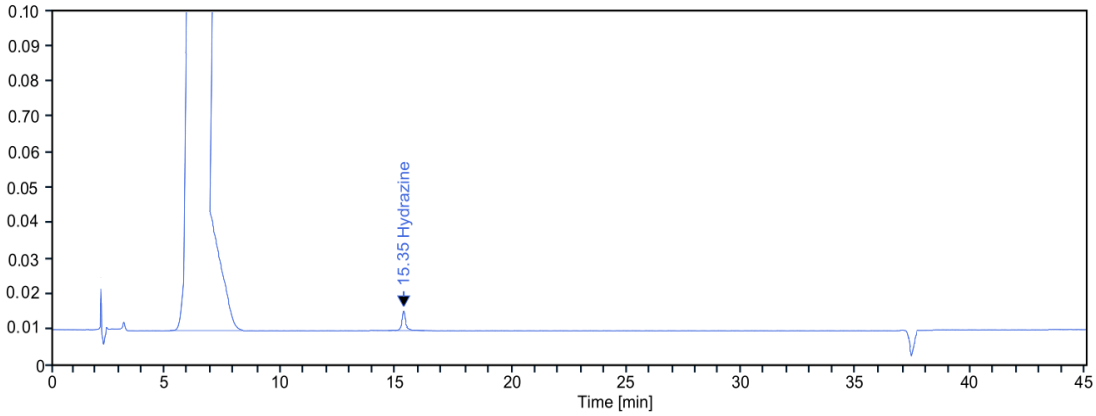


Figure 8: Typical chromatogram of LOQ sample

Table 2 illustrates that the %RSD of peak area for hydrazine standard was found to be 4.98% which is below 10.0% indicating that the system gives precise result.

Method precision: In the method precision, prepare six control samples and six samples by spiking impurity at pecification level and analyse them as per the test method. The samples were prepared as per the method and the precision study results are tabulated in table 3.

Limit of Quantization (LOQ) and Limit of Detection (LOD): A solution containing LOQ and LOD concentrations 0.21µg/mL and 0.069 µg/mL of hydrazine was injected six times.

The %RSD areas of hydrazine standard were calculated. The results of the LOQ precision study are tabulated in table 4.

The Limit of Quantization values obtained for hydrazine are within the acceptance criteria. %RSD of LOQ areas for hydrazine was found to be 4.34% and signal to noise found to be 15.

Table 1
Specificity results

S.N.	Name	Retention Time (min)	Blank	Placebo
1	Blank	ND	NA	NA
2	Placebo solution	ND	NA	NA
3	Standard solution	15.42	No	No
4	Sample solution	ND	NA	NA
5	Spiked Sample solution	15.68	No	No

Table 2
System precision results

S.N.	No. of injections	Peak area of Hydrazine
1	Injection-1	23145
2	Injection-2	20896
3	Injection-3	22884
4	Injection-4	21997
5	Injection-5	20355
6	Injection-6	22001
Avg.		21880
Std.Dev.		1089.077714
%RSD		4.98

Table 3
Method precision results

S.N.	No. of Preparations	% w/w Control samples	% Recovery Spiked samples
1	Preparation 1	ND	96.4
2	Preparation 2	ND	99.1
3	Preparation 3	ND	100.3
4	Preparation 4	ND	97.5
5	Preparation 5	ND	100.9
6	Preparation 6	ND	98.6
Average		NA	98.8
SD		NA	1.6876
%RSD		NA	1.71

Table 4
LOQ precision results

S.N.	Hydrazine
1	6135
2	6288
3	6155
4	6859
5	6225
6	6483
Avg.	6358
Std. Dev.	275.7359
%RSD	4.34

Linearity: The linearity of detector response for hydrazine impurity was demonstrated by preparing solutions over the range of LOQ level to 150% level of target concentration level. A plot of concentration vs. area response of peak was done. The correlation co-efficient between concentration and area response was evaluated. he linearity results for hydrazine in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

Accuracy: The accuracy of the test method was demonstrated by preparing recovery samples at LOQ, 100 % and 150 %of the target concentration level. The recovery

samples were prepared in triplicate for each concentration level. The above samples were injected and the percentage recovery of each sample was calculated for the amount added. Evaluate the precision of the recovery at each level by computing the % relative standard deviation of triplicate recovery samples results. The data obtained are given in table 6 and the method was found to be accurate.

Table 5
Linearity studies for Hydrazine

S.N.	Linearity Level	Concentration (ppm)	Area response
1	25	0.21	5489
2	50	0.41	11087
3	75	0.62	16574
4	100	0.82	21945
5	125	1.03	27391
6	150	1.23	32896
Correlation coefficient (r^2)			0.9999
Slope			26718.8831
Intercept			-7.2625
% Y-intercept			-0.03

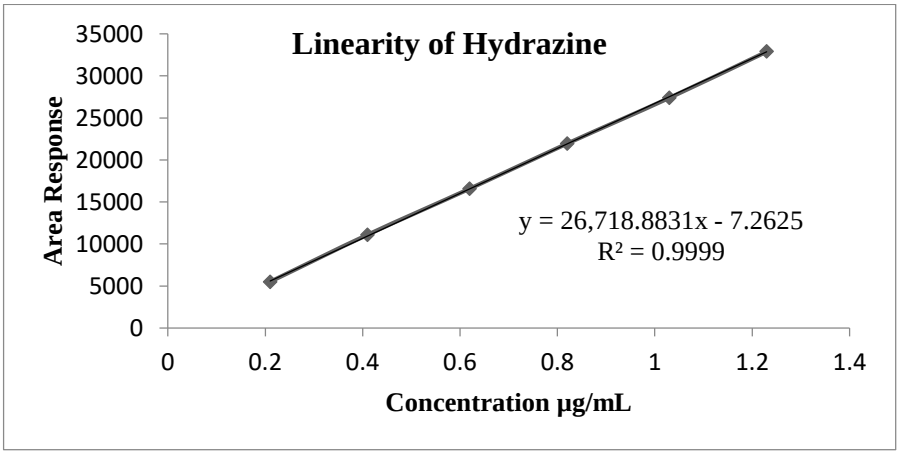


Figure 9: Linearity graph of Hydrazine

Table 6
Recovery studies for Hydrazine

% Level	% found	% Spiked	% Recovery	Mean % Recovery	% RSD
LOQ Level-1	0.0261	0.0263	99.2	100.6	1.21
LOQ Level-2	0.0269	0.0265	101.5		
LOQ Level-3	0.0264	0.0261	101.1		
100% Level-1	0.111	0.103	107.8	104.8	4.74
100% Level-2	0.108	0.109	99.1		
100% Level-3	0.113	0.105	107.6		
150% Level-1	0.153	0.152	100.7	99.8	2.68
150% Level-2	0.157	0.154	101.9		
150% Level-3	0.152	0.157	96.8		

Table 7
Solution stability of standard

Time Interval	Similarity factor	
	Room temperature	Refrigerator
Initial	NA	NA
24hrs	1.05	1.04
48hrs	1.05	1.05

Table 8
Solution stability of sample at Room Temperature and in Refrigerator

Time Intervals	Room temperature	% Difference	Refrigerator	% Difference
Initial	ND	NA	ND	NA
24hrs	ND	NA	ND	NA
48hrs	ND	NA	ND	NA

Table 9
Solution stability of Spiked sample at Room Temperature and in Refrigerator

Time Intervals	Room temperature	% Difference	Refrigerator	% Difference
Initial	0.11	NA	0.11	NA
24hrs	0.13	0.02	0.12	0.01
48hrs	0.15	0.04	0.13	0.02

Table 7 illustrates that the accuracy at LOQ level, 100% level and 150% level for Hydrazine is meeting the acceptance criteria. From the above results, it is concluded that method is accurate.

Solution stability of analytical solutions: Solution stability of standard, sample and spiked sample solutions was established at various conditions such as bench top on room temperature and at refrigerator 2-8°C. The stability of standard, sample and spiked sample solutions was determined by comparison of initially prepared standard, sample and spiked sample solutions with freshly prepared standard solution. The data obtained is given in tables 7 and 9. Solution stability of standard, sample and spiked sample solutions is stable up to 48 hours on both bench top and in refrigerator 2-8°C condition.

Discussion

A simple, economic, accurate and precise RP-HPLC method for determination of hydrazine impurity in ursodeoxycholic acid was developed and validated as per ICH guidelines. In the specificity study there is no interference of diluent and placebo at hydrazine impurity peak. The elution order and the retention time of hydrazine impurity obtained from individual standard preparation and spiked sample preparations are comparable. For system precision studies, six replicate injections were performed. %RSD was determined from the peak areas of hydrazine standard. The results were found to be within the acceptance limits.

For method precision studies, six samples were performed. % relative standard deviation for impurity was found for each set of samples. The results were found to be within the acceptance limits. The limit of detection (LOD) and limit of quantization (LOQ) for hydrazine standard are 0.069 µg/mL and 0.21µg/mL respectively. The linearity results for hydrazine impurity in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99. Calibration curve was plotted and correlation co-efficient for hydrazine impurity was found to be 0.9999 respectively.

The accuracy studies were shown as % recovery for hydrazine impurity at LOQ to 150% level. The limit of % recovered shown is in the range of 80% to 120% and the results obtained were found to be within the limits. Hence the method was found to be accurate. Solution stability parameter was established. Standard, sample and spiked sample solutions are stable up to 48 hrs on both bench top and in refrigerator (2-8°C) condition.

Conclusion

The proposed RP-HPLC method that can quantify genotoxic impurity hydrazine in ursodeoxycholic acid at trace level concentration has been developed and validated as per ICH guidelines. The effectiveness of the method was ensured by the specificity, precision, linearity LOD, LOQ and accuracy. Hence, the method suits well for their intended purposes and can be successfully applied for the release testing of ursodeoxycholic acid into the market.

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