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Short communication

A validated RP-HPLC method for quantitative determination of related impurities of ursodeoxycholic acid (API) by refractive index detection

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ABSTRACT

An isocratic RP-HPLC method was developed and validated for quantitative determination of ursodeoxycholic acid (UDCA) and its related impurities. Considering the lower molecular absorptivity of UDCA, refractive index detector was used to detect the impurities on a Phenomenex Luna C_{18}, 150 mm × 4.6 mm, 5 μm column. The mobile phase was 0.1% acetic acid/methanol (30:70, v/v) and flow rate was 0.8 ml/min. The detector and column temperature was maintained at 40 °C. The method is linear over a range of 0.25–3.5 μg/ml for all impurities and coefficient of correlation (r^2) was ≥0.9945. The accuracy of method demonstrated at three levels in the range of 50–150% of the specification limit and recoveries were found to be in the range of 97.11–100.75%. The precision for all related impurities was below 3.5% R.S.D. The method was applied to commercial bulk drug sample for assay purpose.

1. Introduction

Ursodeoxycholic acid (UDCA); (3-alpha,5-beta,7-beta)-3,7-dihydroxycholan-24-oic acid is a naturally occurring bile acid found in humans in minute quantity [1,2]. It prevents billiary secretion of cholesterol and also diminishes cholesterol intestinal absorption [3,4]. It is used to dissolve cholesterol rich gallstones in patients with functioning gallbladders [5,6] and also in treatment of billiary cirrhosis [7], viral hepatitis [8,9], cystic fibrsosis, etc. [10]. UDCA is most commonly administered therapeutic agent. The potential impurities present in raw material include other bile acids such as lithocholic acid (LCA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) [11,12] causing hepato-toxicity. Other impurities are ursocholic acid (UCA) and cholic acid (CA). The reported impurities are noxious in very small quantities, therefore a rapid, accurate, sensitive method required to control the impurities in bulk drug of UDCA. The bile acids have weak molar absorptivity; the HPLC technique suffers from limited sensitivity [11] and requires the selection of short UV wavelengths [12] which results in increased interference from other constituents. The reported methods describes application of various detection techniques for determination of UDCA and these are high performance liquid chromatography-electrochemical detection (HPLC-ED) [13], derivatization technique [14], thin layer chromatography (TLC) [15], evaporative light scattering detection (ELSD) [16], high performance-capillary electrophoresis (HP-CE) [17], etc. A disadvantage associated with HPLC-ED is the complexity of apparatus, procedure and requirement of high pH (above 13) of eluent with high electrode maintenance [18,19]. TLC is not accurate while ELSD does not produce rectilinear results [20]. This paper describes application of refractive index detector coupled with HPLC to determine UDCA and its related impurities within a time span of 22 min. This method does not have any complexity of apparatus, no additional time consuming derivatization step, no high pH requirement and no more expensive detector like ELSD needed for analysis.

Recently published United States Pharmacopoeia-33 (USP 33) described the UDCA assay procedure on HPLC with refractive index detection using acetonitrile as organic solvent and related substances of UDCA were determined on thin layer chromatography. Other latest European Pharmacopoeia 6 (Ph. EUR.6) and British Pharmacopoeia 2010 (BP 2010) also described the thin layer chromatography method for determination of related impurities of UDCA. In comparison of all Pharmacopoeial methods, this paper presents the HPLC method which determines the related impurities of UDCA up to 0.05% level (LCA, CDCA, and DCA) and 0.10% (CA and UCA) levels using methanol as organic solvent with refractive index detector. Methanol is more cost effective in comparison of acetonitrile used in USP 33 assay method. Comparing to existing Pharmacopoeial TLC methods for related substances of UDCA, this HPLC method is more sensitive (detection up to 0.05% level), faster (separation within 22 min), economical (methanol rather than acetonitrile) and accurate (97.11–100.75) to determines the related impurities for quantitative and qualitative purpose during bulk

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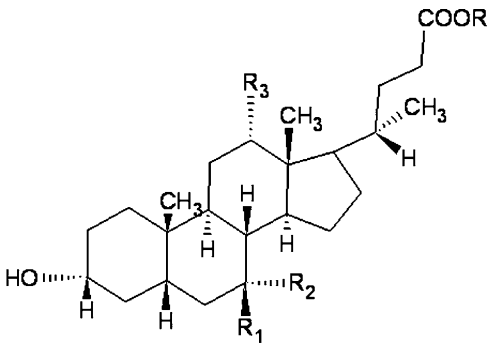

R	R_1	R_2	R_3	API and impurities	Mol. Wt.
H	OH	H	H	UDCA (API)	392.6
H	H	H	H	LCA	376.57
H	H	OH	OH	CA	408.56
H	H	H	OH	DCA	392.6
H	OH	H	OH	UCA	408.56
H	H	OH	H	CDCA	392.6

Fig. 1. Structures of ursodeoxycholic acid and its related impurities.

drug production of UDCA. The structures of related impurities are depicted in Fig. 1.

2. Materials and methods

2.1. Chemicals

Ursodeoxycholic acid and related impurities were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). HPLC grade solvents were supplied from Merck Specialities Private Limited (Mumbai, India). All other chemicals were of analytical grade and purchased from K.J. Enterprises, Mumbai (India). Water used was Milli-Q grade generated in-house on Milli-Q ultrapure water system. Commercial bulk sample (Ursodeoxycholic acid) received from Sun pharmaceuticals Ltd. Jammu (India).

2.2. Equipment

A high-performance liquid chromatographic (HPLC) system of Waters Alliance 2695 consisting quaternary LC pump, vacuum degasser, refractive index detector was used for the study. The output signal was monitored and processed using EmpowerPro data software run on HP computer-Windows-2003 Professional.

2.3. Chromatographic conditions

The chromatographic column used was a Phenomenex Luna, 150 mm × 4.6 mm RP_{18} column with 5 μm particles. The mobile phase was 0.1% acetic acid/methanol with composition of (30:70, v/v) and flow rate was 0.8 ml/min. The column temperature was maintained at 40 °C and the detection was monitored by Waters 2414 refractive index detector at 40 °C. The injection volume was 20 μl. A mixture of mobile phase 0.1% acetic acid/methanol 50:50 (v/v) was used as a diluent to prepare the final concentration prior to inject into the HPLC.

2.4. Preparation of stock solutions

Stock solutions of 5.0 mg/ml UDCA and 500 μg/ml of impurities (LCA, CDCA, DCA, UCA and CA) were prepared in methanol separately.

2.5. Method validation

2.5.1. Linearity

Linearity of UDCA at seven concentration levels from 5% to 150% of analyte concentration (25, 50, 100, 150, 200, 250, 500 and 750 μg/ml) and of each impurity from LOQ to 600% (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg/ml) were evaluated. Linearity was based upon the response factor which was calculated from amount to area ratio of respective peaks. The peak area versus concentration data was performed by least-squares linear regression analysis. Linearity test was performed for 3 successive days within entire concentration ranges.

2.5.2. Accuracy

Standard mixtures containing UDCA and five impurities were prepared and analyzed by HPLC using optimal separation conditions. The accuracy of the method was checked for three different impurity concentration levels (relating to nominal one): 50%, 100% and 150%, by standard addition technique. All impurities were repeated six times and recoveries and percentage R.S.D. were calculated.

2.5.3. Precision of impurities

The precision was evaluated by carrying out six replicates of three concentration levels from entire range of UDCA. The percentage of R.S.D. of six assay values was calculated. The precision of the impurities was test out by injecting six individual preparations of each impurity at three different levels (50%, 100% and 150%). The percentage R.S.D. of area for each impurity was calculated.

2.5.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for impurities were determined at a signal-to-noise ratio of 3 and 10 respectively, by injecting a sequence of dilute solutions with known concentration. Precision study was also carried at the LOQ level by injecting six replicates and percentage R.S.D. of the area was calculated.

2.5.5. Robustness

Robustness of the HPLC method was determined by analysis of samples under purposely altered conditions *i.e.* flow rate and detector temperature. The effect on retention time and peak parameters was studied.

2.5.6. System suitability

The system suitability parameters were defined with respect to theoretical plates, tailing factor, repeatability and relative retention time of the ursodeoxycholic acid peak and its impurities (LCA, CDCA and DCA) in sample solution.

2.5.7. Specificity

Specificity is the ability of the method to measure the impurity response in the presence of its active pharmaceutical ingredient (API). The specificity of the developed LC method for ursodeoxycholic acid was determined in the presence of its impurities LCA, CDCA, DCA, UCA and CA. Relative retention time and response factor calculated for each impurity.

3. Results and discussion

3.1. Method development and optimization

The method development started with RP-C_8 column using methanol and acetonitrile in isocratic mode. The temperature of column and detector was maintained at 40 °C. It was observed that peak shape and symmetry of UDCA, DCA and CA was inappropriate with splitting of peak under the conditions (Chromatogram

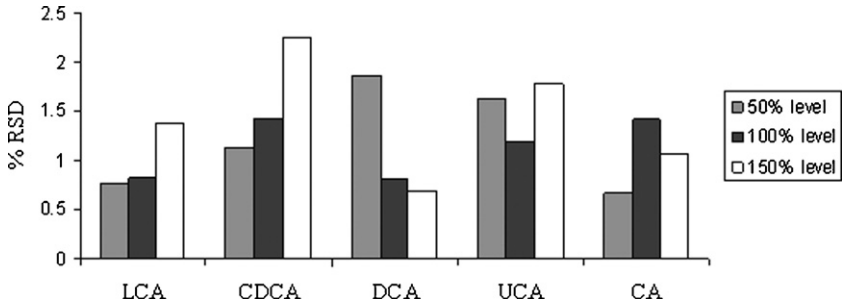

Fig. 2. Accuracy of impurities at 50%, 100% and 150% level.

not shown). Different stationary phases (RP-C_8 and RP-C_{18}) and columns of different dimensions (100, 150 and 250 mm) were evaluated to obtain good peak shape, symmetry and resolution. The following mobile phases were assessed for optimization: (1) KH_2PO_4 (15 mM, pH 4.5) and methanol (60/40, v/v), (2) methanol–acetonitrile–0.02 M aqueous sodium acetate (50:20:30, v/v/v) adjusted to pH 4.3 with phosphoric acid, (3) sodium acetate buffer (15 mM, pH 4.2)–methanol–acetonitrile (30:50:20, v/v/v), (4) methanol–0.1% acetic acid (70: 30, v/v). The mobile phase 1 resolved UDCA, UCA and LCA but did not resolve the impurities CDCA and DCA. The mobile phase 2 showed distorted peak of LCA along with merging of CDCA and DCA peaks. The RP-C_8 column (100 mm length) results very poor peak shape and symmetry of LCA, CDCA and DCA with mobile phase 3, but the best separation of UDCA and impurities was obtained with mobile phase 4 using column RP-C_{18} of 150 mm length with excellent peak shape. The high performance of the RP-C_{18} column of 150 mm length increased selectivity and sensitivity. At last optimum resolution, peak shape and symmetry of UDCA with tailing factor less than 1.5 observed in mobile phase methanol and 0.1% acetic acid in water (75:25, v/v%) but CDCA, DCA and UDCA were very close to each other. When polarity of mobile phase increased, resolution among CDCA, DCA and UDCA was increased significantly (Fig. 2). The quantitative analysis was carried out with the RP-C_{18} column using mobile phase 4. The experimental data: retention times, peak areas and widths, temperature, column information and instrumental data, were entered into EmpowerPro data software.

Table 1
Retention time and response factors of impurities.

Impurity	Retention time	Response factor	Tailing factor
Lithocholic Acid	4.35	1.126	1.25
Deoxycholic acid	5.52	1.125	1.29
Chenodeoxycholic acid	6.76	1.120	1.36
Cholic acid	13.39	Below 3.5	1.33
Ursocholic acid	17.69	Below 3.5	1.41

3.2. Method validation

3.2.1. Linearity

Linear calibration plot for UDCA was obtained over the calibration ranges tested, *i.e.* 25–750 μg/ml and the correlation coefficient ($r^2 \geq 0.9996$). Linearity for impurities was determined from LOQ (0.05%) to 0.7% for LCA, CDCA, DCA, UCA and CA. The correlation coefficient obtained was greater than 0.997. The response factor is within the 5% limit (Table 1) for UDCA and for impurities with described concentration ranges. The results showed that an excellent correlation existed between the peak area and concentration of impurities ($r^2 \geq 0.9945$).

3.2.2. Accuracy of impurities

Accuracy of impurities was evaluated at 0.05%, 0.1% and 0.15% level of 0.5 mg/ml UDCA. The percentage recovery of LCA, CDCA, DCA, UCA and CA acid in bulk drug samples ranged from 97.11 to 100.75 given in Table 2. The deviation from the spiked value is not

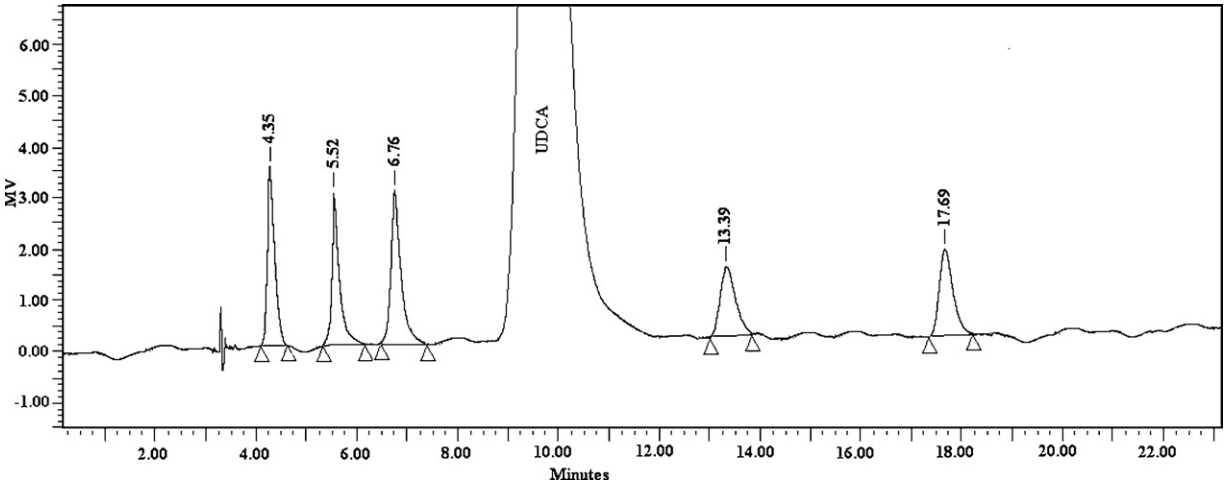

Fig. 3. UDCA spiked with 0.1% level impurities.

Table 2
Accuracy of the method.

Impurity	Spiked value (μg/ml)	Amount recovered (μg/ml)	Mean recovery [%R.S.D.][a]
LCA	0.25	0.2495	99.83 [0.77]
	0.50	0.5003	100.06 [0.83]
	0.75	0.7538	99.35 [1.37]
CDCA	0.25	0.2492	99.72 [1.13]
	0.50	0.5117	102.35 [1.42]
	0.75	0.7538	100.51 [2.25]
DCA	0.25	0.2502	100.10 [1.87]
	0.50	0.4984	99.68 [0.81]
	0.75	0.7556	100.75 [0.69]
UCA	0.25	0.2440	97.62 [1.63]
	0.50	0.4889	97.79 [1.18]
	0.75	0.7471	99.63 [1.78]
CA	0.25	0.2472	98.89 [0.67]
	0.50	0.5004	100.09 [1.41]
	0.75	0.7283	97.11 [1.07]

[a] Mean values represent six replicates of each concentration.

Table 3
LOD and LOQ for impurities.

Impurity	LOD	S/N	LOQ	S/N
LCA	0.016%	2.8	0.05%	9.5
CDCA	0.016%	2.3	0.05%	10.1
DCA	0.016%	3.5	0.05%	9.6
UCA	0.03%	3.6	0.10%	10.4
CA	0.03%	2.8	0.10%	10.7

Table 4
Robustness test results of UDCA and its impurities.

Parameter changed	Deviation of amounts for UDCA (% R.S.D.)	Resolution between UDCA and DCA	Deviation of amounts (%) for impurities spiked amounts)
Detector			
Temperature	1.21 ± 5%	1.93 for −5% change	
		1.87 for + 5% change	<5% R.S.D. for amounts
Flow	1.89 ± 0.1 ml	1.89 for + 0.1 ml change	<5% R.S.D. for amounts
		1.95 for −0.1 ml change	

Table 5
Typical system suitability results.

Compound	RT	Plate no.	Tailing factor	RRT
LCA	4.35	5273	1.35	0.43
UDCA (API)	9.98	12839	1.51	1.0
CDCA	5.52	7289	1.46	0.55
DCA	6.76	8827	1.44	0.67

Table 6
Analysis of a commercial UDCA drug sample.

Impurity	Retention time	Impurities found (%)
LCA	4.52	–
CDCA	5.35	–
DCA	6.76	–
CA	13.76	–
UCA	17.66	0.14
	Total impurities	0.14
	Assay %	99.86
	R.S.D.	1.23

more than ±5% as depicted in Fig. 2. The maximum deviation is ±2.25% for the determined concentration. HPLC chromatogram of 0.1% level of related impurities in ursodeoxycholic acid bulk drug sample is shown in Fig. 3.

3.2.3. Precision of retention times and areas of impurities

The percentage R.S.D. of area and retention time of UDCA impurities was within 3.5% confirming high precision of the method.

3.2.4. Limit of detection and limit of quantitation

The limit of detection of LCA, CDCA, DCA, UCA and CA were 0.016%, 0.016%, 0.016%, 0.03% and 0.03% (of analyte concentration, *i.e.* 500 μg/ml), respectively for 20 μl injection volume as depicted in Table 3. The limit of quantification of LCA, CDCA, DCA, UCA and CA were 0.05%, 0.05%, 0.05%, 0.10% and 0.10% (of analyte concentration, *i.e.* 500 μg/ml), respectively. The precision at LOQ concentration for all impurities was below 10%.

3.2.5. Robustness

In all purposely altered chromatographic conditions as per Section 2.5.5 (flow rate and detector temperature), the resolution between closely eluting impurities, namely CDCA and DCA was greater than 1.5 maintained, demonstrating the robustness of the method (Table 4). The parameter changes cause deviations for the areas of less than 2%, which is acceptable for ursodeoxycholic acid.

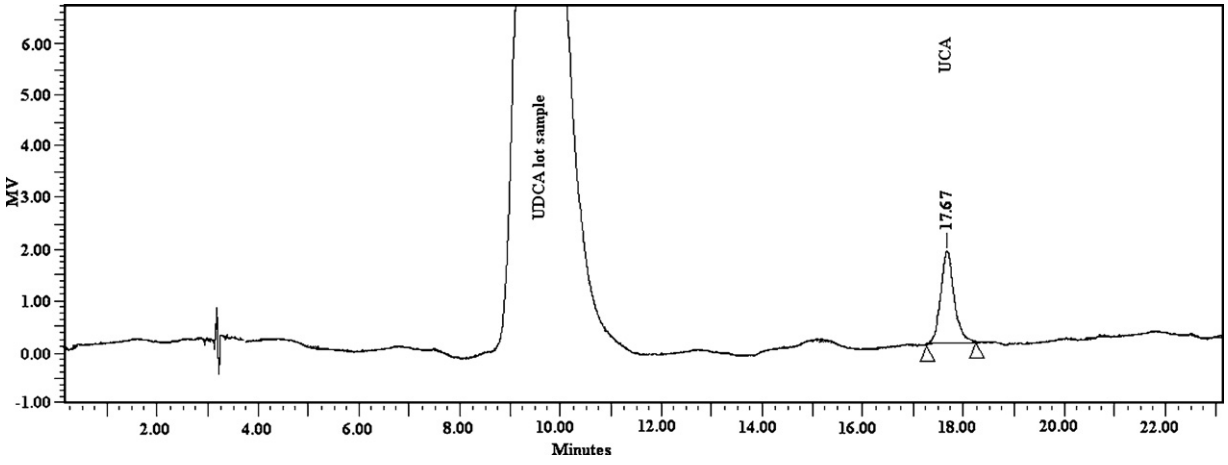

Fig. 4. Commercial UDCA lot sample showing UCA impurity (0.14% level).

The results for the impurities are also shown in Table 4. All results are within the 10% limit for the areas.

3.2.6. Range of impurities

The range of impurities with an acceptable precision, accuracy and linearity is between 0.25 μg/ml to 3.0 μg/ml (LCA, CDCA, DCA impurity) and 0.25 μg/ml to 3.5 μg/ml for (UCA, CA impurity).

3.2.7. Carryover effect

The carry-over was evaluated by injecting the stock solution 6 times followed by the injection of diluent used for sample preparation. The carry-over was found ≤0.012% (Table 5).

3.2.8. Analysis of commercial UDCA sample

A commercial UDCA sample analyzed with the proposed method and UDCA assay was found to be 99.86 with 1.23 percentage R.S.D. Only one impurity ursocholic acid (RT: 17.67) was found which was ≤0.14% (Fig. 4 and Table 6).

4. Conclusion

An HPLC method was developed and validated for the determination of ursodeoxycholic acid and its related impurities using refractive index detection technique. The method is rapid, practical, robust, sensitive, specific, accurate and reliable for analysis of related impurities of ursodeoxycholic acid. The base line separation was found greater than 1.5 with no matrix interference across the elution windows among the peaks of interest. The method has been successfully used to quantify impurities (0.14%) in commercial ursodeoxycholic acid bulk sample.

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