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Development and Validation of RP-LC-UV Method for Determination of Ursodeoxycholic Acid in Capsule and Human Serum

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Abstract: Here we have depicted a reverse phase high performance liquid chromatographic novel specific, precise and accurate, method that has been developed and validated for determination of Ursodeoxycholic Acid in pharmaceutical dosage forms of capsules and in human serum. Acetonitrile: Methanol: D.I water pH 2.4±0.1 was the mobile phase at flow rate 1.0 ml/min using a Hibar® Lichrosorb RP-18 (10µ) column monitored at wavelength of 220nm. The calibration curve was linear with a correlation coefficient more than 0.9995 for the drug with a linearity range between 300ng/ml -1000µg/ml. The averages of the absolute and relative recoveries were found to be 99.69% with 1000ng/ml limit of quantification and 300ng/ml limit of detection. The developed method was validated in accordance to ICH guidelines. The projected High-performance liquid chromatographic method was successfully applied to quantify the amount of Ursodeoxycholic Acid in bulk and commercial dosage forms and human serum samples with efficient recovery.

Key words: RP-HPLC · ICH · Stability · API's

INTRODUCTION

Ursodeoxycholic Acid is (UDCA) is a white, odorless, crystalline powder with a bitter taste. Chemically it is (3α, 5β, 7β)-3, 7-dihydroxycholan-24-oic acid (Fig. 1). It is water insoluble and used as a drug for the dissolution of cholesterol gallstones [1-3]. As it has the ability to reduce the cholesterol saturation of bile [4] therefore it is therapeutically applicable bile acid widely used for the dissolution of cholesterol-rich gallstones and in the treatment of chronic liver diseases associated with cholestasis [5]. The use of UDCA for treatment of other liver diseases, such as primary biliary cirrhosis, chronic hepatitis and biliary pains has also been demonstrated [6-11].

Some methods are available for UDCA analysis in monographs such as titrimetric method is official in British and European Pharmacoepia [12, 13], similarly in USP [14] monograph HPLC method is available with utilization of RID. Additionally several papers have been described for determination of UDCA by HPLC with UV detection [5, 15, 16], while many methods are available with use of expensive instruments or methodology which are usually not available for routine laboratory analysis, for example using RI detector [15, 17] and others have used complexometric methods [18]. The other methods include capillary electrophoresis with indirect UV detection [19] TLC-densitometry [20] and some have used biological methods [21, 22].

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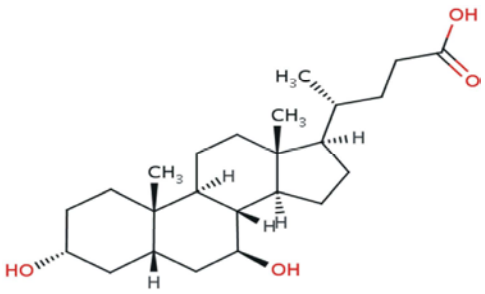

Fig 1: Chemical structures of Ursodeoxycholic Acid

To the best of our research survey till now no such method is available which can determine UDCA efficiently with facile and simplicity of methodology, therefore an endeavor was made to develop a simple, sensitive and validated RP-HPLC method using UV detection for the estimation and routine analysis of UDCA in bulk drug, in commercially available dosage forms and which provides high sensitivity for routine use and diminishing the time of sampling and chromatographic analysis.

The proposed HPLC assay for determination of UDCA has not yet been reported and therefore, this study claims to be a novel one. The applicability of the method was confirmed for analysis in pharmaceutical products and results of analysis were validated in accordance with ICH guidelines [23].

Experimental: The present method was designed to be easy to use, sensitive, rapid and simple sample preparation for active ingredient. Isocratic elution was selected for quantitation in pharmaceutical formulation and blood samples.

Material and Reagents: UDCA was kind gift from National Pharmaceuticals, whereas phosphoric acid, Acetonitrile (HPLC grade), Methanol (HPLC grade) were purchased from Merck (Germany). The pharmaceutical dosage forms containing UDCA were obtained from commercial source including Urso Capsules 250mg/Caps. (AGP (pvt) ltd.), Ursogal® capsules. (Shrooq Pharmaceutical (pvt) ltd.), Triptore® capsules (Consolidated chemical laboratories (pvt) ltd.). RO plant (Waterman, Pakistan) was used to obtain distilled water and 0.45µm membrane filter (Millipore, Bedford, MA, USA) was utilized for further filtration purpose.

Instruments: Chromatography was performed on a Hibar® LichrosorbRP-18 HPLC column, (4.6 x 250 mm; 10 µm bead size) maintained at 25°C. A UV-visible Shimadzu 1650 PC spectrophotometer with UV Probe software, ultrasonic cleaner (Elmasoni E 60 H), Jenway 3240 pH meter and Sartorious TE2145 analytical balance were used in the research work. For chromatography a SIL 10A auto injector HPLC system comprising of SCL 10A system controller, SPD 20A prominence UV/VIS detector and Shimadzu LC 20 AT pump with LC Solutions software was used.

Chromatographic Conditions: HPLC analysis was carried out at ambient temperature. The compound was chromatographed isocratically with a mobile phase consisting of Acetonitrile (HPLC grade): Methanol (HPLC grade): Distilled water (30:50:20 v/v/v) with pH adjusted to 2.4±0.1 (if required) using phosphoric acid 0.5M, while Methanol (HPLC grade) was used as dilution solvent. A 0.45µm membrane filter (Millipore, Bedford, MA, USA) was used to filter mobile phase, standard, sample and other solutions. Flow rate of chromatographic system was maintained steady as 1.0 ml/min and injected volume was kept constant 20µL with a 20µL loop. The effluent was monitored spectrophotometrically at wavelength of 220nm.

Analytical Procedure

Standard Preparation: In a 100ml volumetric flask, accurately 100 mg of UDCA was weighed and dissolve in 70ml of dilution solvent sonicated for 5 minutes and then added up to the mark with dilution solvent to get stock solution-A containing 1000µg/ml of UDCA and was used in further analytical steps. For working standard preparation 10ml of stock solution-A was diluted in 20ml flask to get working solution of 500µg/ml and was filtered through 0.45µm filter followed by injecting into the HPLC system.

Sample Preparation

Sample Preparation for Products: For making a sample from commercially available capsules, 10 capsules were opened and content was weighed individually and then mixed to get an evenly homogenized sample, weighed the sample accurately equivalent to 100mg UDCA taken in 100ml volumetric flask and 70 ml of diluent was added. The sample was sonicated for 05 minutes, added diluent up to the mark and placed on stirrer for 10 minutes. For working sample preparation 10ml of stock sample solution was diluted in 20ml flask to get working solution of 500µg/ml UDCA. Prior to injcting into HPLC system solution was filtered through 0.45µm filter.

Sample Preparation for Serum: Serum sample was prepared as described earlier [24], briefly healthy volunteers were involved in study for drawing their blood with help of trained medical technician, in evacuated glass tube and processed. For making working sample 10ml of stock solution-A was taken in 20ml flask, followed by 5ml of serum. The sample thus obtained was stirred for 10 minutes and then diluted up to the mark and was filtered through 0.45µm filter before injecting into the HPLC system. For analysis in serum, each working solution was prepared and analyzed in triplicate.

Stability Studies: Environmental chambers were used for stability evaluation of commercially available samples (capsules). For this purpose samples were placed at ambient conditions of 30°C temperature with 65% relative humidity and accelerated conditions of temperature that is at 40°C with 75% relative humidity in environmental chamber for six months as guided by ICH [23]. A stability protocol was followed for six months and assays were made as described earlier.

Method Validation: Method validation was performed following the ICH guideline [23] according to which various parasmeters were investigated including range, linearity, accuracy, specificity, intra-day and inter-day precision etc.

To study linearity of method for standard solutions, several dilutions were prepared from stock solution-A to give standard solutions in range of 300ng/ml -1000µg/ml of drug content. Standard calibration curve was generated using regression analysis. For specificity commonly used excipient in dosage forms preparation were spiked in a pre-weighed quantity of drug and then peak areas were measured and calculations done to determine the quantity of the drug recovered.

Precision was done by analyzing corresponding standard daily for a period of three days *i.e*. Inter-day precision and three times a day with 08 hours interval (Intra-day precision) against freshly prepared standard solutions. For determining accuracy the reference standards were accurately weighed and added to the sample, to get three different concentration levels *i.e*. 110% -120% -130% of the APIs. At each level, solutions were prepared in triplicate and recovery percentage was determined.

Standard solutions were sequentially diluted to get concentrations ranging from 1000ng/ml to 300ng/ml in order to establish limit of detection and quantification (LOD & LOQ) for the method and injected onto the chromatographic system. Limit of detection was defined as the concentration for which a signal-to-noise ratio of 3 was obtained and, for quantification limit; a signal-to-noise ratio of 10 was considered.

For studying robustness of method, sample solutions were analyzed while deliberately altering different chromatographic parameters of method, such as mobile phase pH, temperature, flow rate etc.System suitability of the chromatographic system was evaluated by analyzing the symmetry of the standard peaks and theoretical plates of the column.

RESULTS AND DISCUSSION

For any drug available in the market to have high quality products, their analytical HPLC method development and its validation are the utmost requirements. Though a few methods are available for determination of the UDCA as described earlier, but many of them are used only for certain definite objectives and none can be generalized for its determination in form of pharmaceutical products and serum. Similarly none of them is as much sensitive as ours is; in terms of its Precision, accuracy, %recovery, limit of detection (LOD) and limit of quantification (LOQ). Moreover this method is sensitive enough to be used for pharmacokinetic studies as its LOD and LOQ are in nanogram range.

Method Development and Optimization: To develop an efficient and purpose to fit method for analysis, several parameters were studied at length, including maxima wavelength, composition of mobile phase, optimum pH and concentration of standard and sample solutions. All the ingredients particularly the API's were diluted in dilution solvent and then run through UV spectrophotometer in UV range of 190nm-400nm to get maximal wavelength, where maximum absorbance was gained *i.e*. 220nm at which the molecule gave a satisfactory absorbance and representable chromatogram. Mobile phase was selected in terms of its constituents and their combination was maintained such that chromatograms comply with all the requirements of an efficient analytical approach. The chromatographic parameters were evaluated using a Hibar® Lichrosorb RP-18 (10µ), the mobile phase composed of Acetonitrile (HPLC grade): Methanol (HPLC grade): D.I water of given proportion which promoted a short run time (10 min) without any interference, so this condition was adopted in subsequent analysis.

Table 1: Calibration curve data and validation parameters

Parameter	Inference UDCA
Linearity range (ng /ml)	300 ng/ml -1000µg /ml
Intercept(c)	1106073
Standard Regression equation (y=mx+c)	
Slope(m)	102.528
Correlation coefficient(R2)	0.999815
Limit of detection (LOD) (ng /ml)	300
Limit of quantification (LOQ) (ng /ml)	1000

Table 2: Inter-day and intra-day precision and recovery studies

Active Drugs	Nominal Concentration (µg /ml)	Day 1	Day 2	Day 3	Mean	Inter-Day RSD%
UDCA	400	99.14%	98.78%	99.86%	99.26%	0.449
	500	100.15%	100.69%	99.93%	100.26%	0.319
	600	100.57%	99.95%	98.18%	99.57%	1.013
	Mean	99.95%	99.81%	99.32%	99.69%	0.997
	Intra-Day RSD%	0.600	0.786	0.809	0.417	

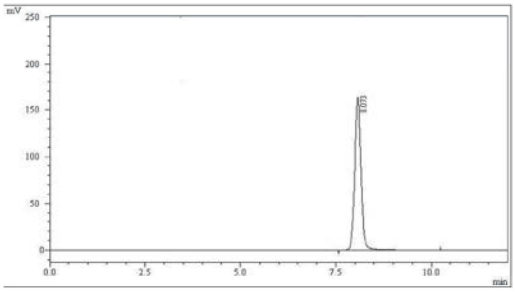

Fig2a- Typical Chromatogram of standard

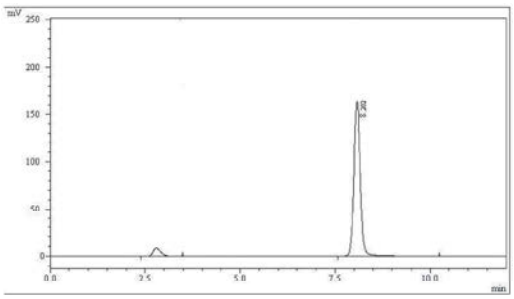

Fig 2b- Typical Chromatogram of product sample

Validation Studies: For validation studies linearity was determined in the range of 0.06%-200% with respect to 500µg/mL. The assay was judged to be linear as the correlation coefficient was greater than 0.9995 calculated by the least-square method. A linear correlation was found between the peak areas and respective concentrations of UDCA, in assayed range. The regression analysis data are presented in Table 1.

Specificity or selectivity of the assayed method is proven, as the chromatogram in standard (Figure 2a) was found identical with Chromatogram of sample shown in Figure-2b and no interfering peak was observed. Peak purities higher than 98.0% were obtained in the chromatograms of sample solutions, demonstrating that other compounds did not co-elute with peak of interest. The chromatogram obtained with the mixture of the product's excipient proves that there is no interference from excipient and peak of interest fulfill all the requirements of symmetrical peak and hence conforms the specificity parameters.

Precision of an analytical method is the degree of coherence among individual test results when the method is applied repeatedly to multiple samples of homogeneous bulk. Intra-day precision of the method was evaluated at three different independent concentrations i.e. 80%, 100% and 120% for the drug (n=3) by determining their assays. Inter-day precision of the method was tested for 3 days at the same concentration levels. Since %RSD of inter-day and intra-day precision was less than 2% *i.e.* 0.997 and 0.417 respectively hence assuring that the proposed method is quite precise and reproducible (Table 2).

Accuracy was investigated by means of addition of reference standards to a mixture of the products excipients. Recovery studies of the drug were carried out for accuracy parameter at three different concentration levels i.e. multiple level recovery studies. A known amount of API standard was added into pre-analyzed sample and subjected to the proposed HPLC method. The mean recovery (n=9) was 98.18%-100.69%, demonstrating the accuracy of the method. Percentage recoveries for marketed products were found to be within the limits (Table 3).

Table 3: Contents of UDCA, in pharmaceutical dosage forms

SAMPLES	Content (%) ± S.D. ---------- UDCA
Urso® Capsules	97.94%± 0.25
Ursogal® Capsules	98.38 %± 0.18
Triptore® Capsules	99.15%± 0.39

S.D. = Standard deviation

Table 4: Robustness of the method

Chromatographic Conditions	Variation	UDCA ---------- Tailing Factor	Retention time
Temprature (ºC)	23	0.675	8.591
	27	0.549	7.853
Flow rate (ml/min.)	0.8	0.763	8.846
	1.2	0.514	7.613
Mobile phase pH	2.6	0.648	8.795
	2.2	0.597	7.314

Table 5: Summary of stability studies

TEST (Claimed content)	INTERVAL ---------- Initial	1Month	2Month	3Month	6Month	Mean	RSD%
Studies at Accelerated (40ºC+75%H)							
Urso® Capsules	99.659	98.113	97.327	96.826	96.591	97.70	1.108
Ursogal® Capsules	100.529	99.974	99.9639	99.482	98.723	99.73	0.605
Triptore® Capsules	99.987	99.182	98.529	97.982	97.793	98.69	0.807
Stability Studies at Long Term (30ºC+65%H)							
Urso® Capsules	99.659	99.593	99.892	99.156	98.839	99.43	0.379
Ursogal® Capsules	100.529	100.1	100.326	99.871	99.895	100.14	0.253
Triptore® Capsules	99.987	99.518	99.195	99.837	99.239	99.56	0.315

The statistical analysis showed no significant difference between results obtained employing the analytical conditions established for the method and those obtained in the experiments in which variations of some parameters were introduced. The parameters used in system suitability test were symmetry of peak, tailing factor, resolution and RSD of peak area for replicate samples. Thus, the method proves to be robust for changes in mobile phase pH, flow rate and column temperature (Table 4).

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions, for example temperature, humidity and light hence enables recommendation of storage conditions, retest periods and shelf life to be established. The two main aspects of drug product that play an important role in shelf-life determination are assay of the active drug and the degradation products generated during stability studies. The proposed assay method was applied to stability study of commercially available products, for which the samples were placed at previously mentioned conditions. Stability study was performed according to stability protocol as in Table 5.

Samples were analyzed and percentage of contents was measured. According to the results obtained UDCA was found to be stable at applied conditions of temperature and relative humidity and were accurately analyzed with the proposed method.

CONCLUSION

The proposed new HPLC method described in this paper provides a simple, convenient and reproducible approach for the identification and quantification of UDCA in bulk, human serum and pharmaceutical formulations with good presentation. In addition, this method has the potential application to clinical research of drug combination. Analytical results are accurate and precise with good recovery and lowest detection limit values. In short, the developed method is simple, sensitive, easy and efficient having small chromatographic time and can be used for routine analysis in QC laboratory and therapeutic monitoring.

Conflict of Interest Statement: It is hereby declared that there is no conflict of interest among authors.

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