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Development and validation of a LC-UV Method Applied to the Quality Control of Ursodeoxycholic Acid in Raw Material and Pharmaceutical Formulations

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Abstract:-A simple, fast, and sensitive HPLC method with UV detection has been developed for the quantitation of ursodeoxycholic acid (UDCA) in raw material and pharmaceutical formulation (suspension). Cromatographic conditions were: Symmetry-C18 column (150 mm x 4.6 mm, id; particle size 5 µm), 40 ˚C, 100 µL injection volume and UV detection at 200 nm. The flow rate was 1 ml/min using acetonitrile - phosphoric acid (pH 3.0; 0.15mM) (48:52) as mobile phase. The method was validated according to international guidelines (ICH guidelines) for specificity, linearity, LOD, LOQ, precision, accuracy, and robustness. The HPLC-UV method was found to be suitable for the quality control of UDCA raw material and pharmaceutical formulation.

Keywords: *ursodeoxycholic acid, HPLC-UV, quality control, pharmaceutical formulation, raw material*

I. INTRODUCTION

Ursodeoxycholic acid (UDCA) (3α, 7β- dihydroxy-5β-cholan-24-oic acid), (Figure 1), also known as ursodiol, is a naturally occurring bile acid (BA). BAs are steroid compounds, hydroxyl derivatives of 5β-cholan-24 oic acid [1]. They have different physicochemical properties according to the number, position and orientation of their hydroxyl groups, and type of conjugation with glycine and taurine, which form the glyco- and tauro- derivatives. These factors influence their properties like solubility, detergency and hydrophobicity. Moreover, primary BA are cholic acid (CA) and chenodeoxycholic acid (CDCA) and secondary BA, deoxycholic acid (DCA), lithocholic acid (LCA), (all of them 3α-position), and UDCA (3β-position) (Fig 1). UDCA is the epimer of CDCA (3α-position) and is also less hydrophobic, detergent and toxic BA due to this structural modification [2].

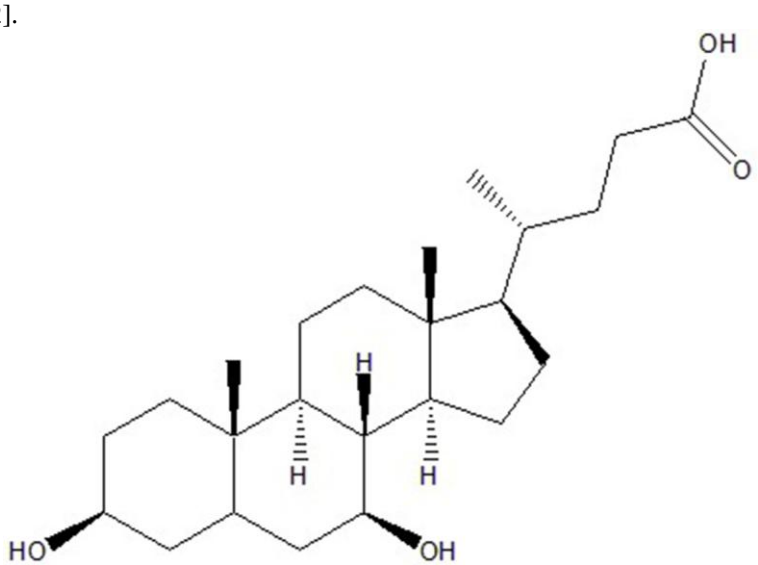

Figure 1. Chemical structure of ursodeoxycholic acid (UDCA)

Thus, UDCA has been used as therapeutic agent for the treatment of hepatobiliary disorders such as cholestasis, biliary dyspepsia, primary biliary cirrhosis and different cholestatic conditions [3], [4].

The UDCA USP official monograph refers to an HPLC method coupled to a differential refractive index detector for the analysis in raw material [5]. The application of this type of detector is due to the low BA absorptivity. Moreover, several analytical methods for the quantification of UDCA in biological fluids and few pharmaceutical dosage forms have been developed. Most of them describe HPLC methods coupled to MS and evaporative light-scattering mass detection in biological matrix [6]–[9]. BA analysis in biological fluids has been performed by GC - MS but these techniques require [10], [11] laborious sample preparation including fractionation, deconjugation and derivatization of free and conjugated BA prior to the analysis [12]. Other method like capillary electrophoresis (CE) has been reported. In this sense, in a previous work, we developed a CE- UV method applied to determination of BA in pharmaceutical formulations [13]. Although CE is suitable for the analysis of BA in pharmaceutical formulations and raw material, it requires a high sample amount and CE equipment is not a common laboratory instrument. As far as we know, there is no report on the use of HPLC – UV for the determination of UDCA in raw material and pharmaceutical formulation. The aim of this work was to develop and validate a simple, fast and sensitive HPLC-UV method for the quantification and quality control of UDCA in raw material and liquid pharmaceutical formulation.

II. EXPERIMENTAL

2.1 Chemicals and Reagents

UDCA, was supplied from Sigma Aldrich (St. Louis, MO, USA). UDCA raw material was supplied from Magel S.A. (Buenos Aires, Argentina). Methanol and acetonitrile were HPLC-grade, phosphoric acid and ammonium acetate were purchased by Merck (Darmstadt, Germany). Methylparaben (Nipagin), propylparaben (Nipasol) and xanthan gum were supplied from Magel S.A (Buenos Aires, Argentina). Ultrapure water was obtained by an EASYpure^{TM} RF equipment (Barnstead, Dudubuque, IA, USA). All solutions were filtered through a 0.45µm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and degassed before use.

2.2 Equipment

The HPLC equipment was a Thermo Scientific SCM1000 with a quaternary pump, P4000 degasser, AS3000 autosampler, thermostatted column compartment and UV2000 detector (Waltham, Massachusetts, USA). Chromatograms were processed using ChromQuest 5.0 software ChromQuest 5.0 software controlling instrumental parameters.

2.3 Chromatographic Conditions

The chromatographic separation was carried out using a reverse phase Symmetry-C18 column (150 mm x 4.6 mm, id; particle size 5 µm), supplied by Waters (Milford, Massachusetts, USA). The mobile phase (MP) contained acetonitrile - phosphoric acid (pH 3.0; 0.15mM) (48:52). Isocratic separation was carried out with an injection volume of 100 µL, the flow rate was set at 1 mL/min and the column temperature was set at 40 ˚C. The UV detection was carried out at 200 nm and all analysis was performed at 25°C. A run time of 8 min was employed for determination of UDCA in raw material and pharmaceutical suspension.

2.4 Standard Solution

2.4.1 UDCA Stock and standard solution

A 500 µg/mL stock solution was prepared in methanol and appropriately diluted in mobile phase to render a 250 µg/mL standard solution.

2.5 Sample solution

2.5.1 UDCA raw material

UDCA raw material was prepared by weighing 50 mg into a 100 mL volumetric flask and dissolving them with methanol. An aliquot of 25 mL of this solution was diluted to 50 mL with mobile phase.

2.5.2 UDCA analysis in pharmaceutical suspension

A UDCA suspension (25 mg/mL) was shaken vigorously by hand immediately before use. 1g was accurately weighed in a 50 mL volumetric flask and dissolved in methanol, sonicated for 5 min and centrifuged for 10 min. A final dilution was performed from the supernatant obtained in the previous centrifugation step, with mobile phase to make a concentration of 250 µg/mL of UDCA.

2.6 Stress Conditions

Oxidation: 5 mL of a 1% w/w hydrogen peroxide solution was added to 100 mg of UDCA accurately weighed and refluxed during 24 hours at 60˚C. Acidic: 100 mg of UDCA with 5 mL of 0.01 M hydrochloric acid solution were refluxed during 24 hours at 60˚C. Alkaline: similar condition to the method described under "acid", using 0.01 M sodium hydroxide solution. Light: A 250 µg/mL UDCA solution was exposed to white light during one week.

III. RESULTS AND DISCUSSION

3.1 HPLC-UV Method Development

Most of the HPLC method reported for the analysis of UDCA in different matrices use C18 column and acetonitrile and buffer solutions at acidic pH values as mobile phase. Thus, the method development started with a RP-C18 column, Thermo (150 mm, 4.6 mm, 5 µm) but a poor peak shape was obtained. Thus a symmetry column was used instead, and an excellent peak shape was obtained. As a consequence, an increased sensitivity and specificity was accomplished. In all cases, the mobile phase was acetonitrile : 0.15 mM phosphoric acid at pH 3.0. The percentage of organic solvent was increased from 40 to 60 to obtain an optimum resolution between excipients and UDCA (Fig. 2)

Column temperature was set at 40˚C. The UV detector was set at 200 nm due to the low BA UV response. However, an adequate sensitivity was obtained with a suitable LOD, LOQ and a baseline resolution compared to an LC- coupled to refractive index detector [5].

In all cases the chromatographic parameters were set to obtain a good shape peaks. Thus the development of the chromatographic method was found to be suitable in terms of efficiency (3990); retention factor (2.94), tailing factor (0.95) and resolution (2.8) according to USP 39 [14].

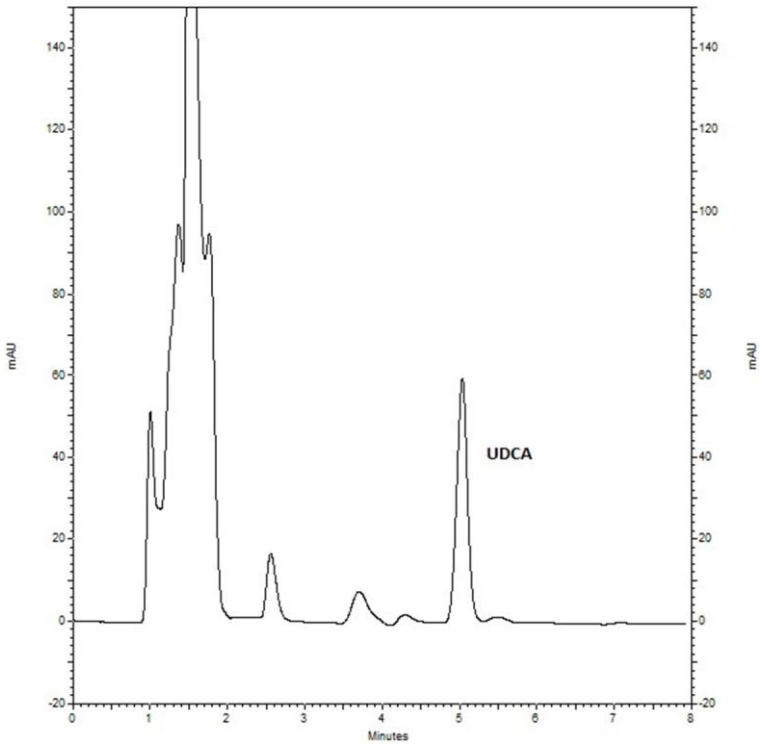

Figure 2. Representative chromatogram of UDCA (250 µg/mL) in liquid pharmacetutical formulation. Experimental conditions are given in the text.

3.2 Validation

The validation of the LC-UV method was carried out according to the International Conference on Harmonization (ICH) guidelines [15]. The following parameters were evaluated: specificity, linearity, LOD and LOQ, precision, accuracy and robustness

3.2.1 Specificity

The specificity of the method was studied by two different ways. First, UDCA was subjected to forced degradation studies in stress conditions (oxidation, acid, alkaline and light). Second, the specificity was investigated by running placebo samples for possible interference. In the first study, UDCA was degraded in the presence of acid and alkali, while exposure to light and oxidation produced minimal degradation of the same. In

the second assay, no excipient interference was observed at UDCA retention time. For all the above mentioned, the method proved to be specific (Fig. 3).

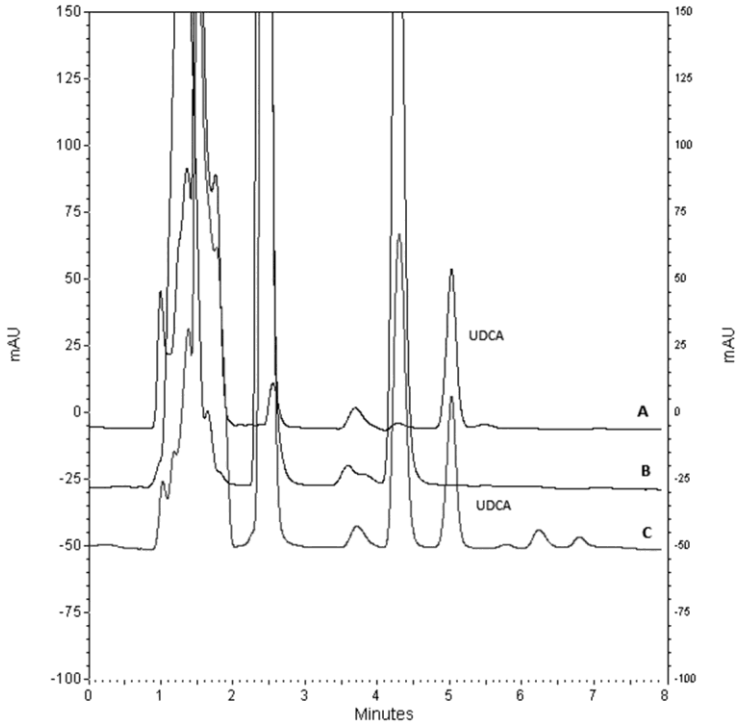

Figure 3. (A) Chromatogram of standard solution of UDCA (250 µg/mL). (B) Chromatogram of blank excipients. (C) Chromatogram of UDCA in suspension formulation. Experimental conditions are given in the text.

3.2.2 Linearity, LOD, and LOQ

Linearity was performed at five UDCA concentration levels (150.0, 200.0, 250.0, 300.0 and 500.0 µg/mL) where each concentration was injected by triplicate. A good correlation was obtained according to international guidelines.

The LOD and LOQ were determined based on signal-to noise ratio of 3:1 for estimating the LOD, whereas a 10:1 ratio was used for the LOQ. The results were 32 and 108 ng/mL of UDCA respectively, on column (Table 1).

Table 1: Linearity, LOD and LOQ, precision and accuracy for UDCA

Parameters	**UDCA**		
Linear range (µg/ml)	150 – 500		
R^2	0.9903		
LOD (µg/mL)	0.32		
LOQ (µg/mL)	1.08		
Precisión (RSD)			
Intra-day (n=6)			
Peak height	0.81		
Migration time	0.14		
Inter-day (n=18)			
Peak height	1.95		
Migration time	0.45		
Accuracy			
Spiked levels**	80%*	100%*	120%*
Raw material Supension	99.8 (0.4) 99.6 (0.5)	100.2 (0.3) 101.3 (0.2)	99.9 (0.4) 99.2 (0.2)

*respect to the label content.

** RSD values between brackets corresponding to n=3.

3.2.3 Precision and Accuracy

Precision was evaluated for intra-day (n=6) and inter-day (n=18) and it was determined as RSD for peak area and retention times (Table 1). A good precision was observed for the developed method. According to data obtained was observed a good precision of the developed method.

Accuracy was evaluated from recovery studies. Placebo samples containing all excipients present in the suspension were supplemented with UDCA at 80; 100 and 120% concentration levels of the nominal values, three replicates of each level were assayed. Percentages of recovery were obtained in the range of 99.2 to 101.3% (Table 1).

3.2.4 Robustness

Robustness was evaluated by the Placket-Burman design with seven parameters at two levels. The seven variables included in the design were: column temperature (A); buffer pH (B); percentage of organic solvent (C); flow rate (D); injection mode (E); detector wavelength (F) and sample sonication time (G). These parameters were selected on the basis of their influence on the analytical method performance (Table 2). The effects of the variables in the mean theoretical plates (N); tailing factor (T), retention factor (K') (14) and UDCA content were evaluated. Statistical analysis was used to determine the effects of each variable and its significance. To show the results, standardized Pareto charts were constructed. As can be seen in figure 4 none of the variables have a significant effect. Data and analyses obtained confirm the robustness of the analytical method.

Table 2: Variables and their levels for robustness test

Selected variables [a]	**Units**	**Abbreviation**	**High level**	**Low level**
Column temperature	˚C	A,a	42	38
pH	-	B,b	3.2	2.8
Organic solvent	%	C,c	50	46
Flow	mL/min	D,d	1.2	0.8
Injection mode	-	E,e	Push	Pool
Wavelenght	nm	F,f	202	198
Sonication time	min	G,g	7	3

*Upper and lower case letter represent high and low level of the variable respectively

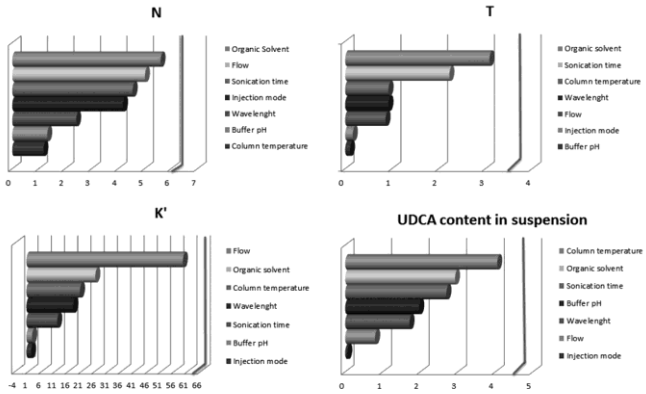

Figure 4. Pareto charts of standardized effects

3.3 Quantitative analysis of UDCA in raw material and suspension

UDCA content in raw material and suspension was determined according to the developed method. The results are in good agreement with the labeled content values of the pharmaceutical formulations (Table 3).

Table 3: Analysis of UDCA in raw material and pharmaceutical formulation

	Label content (%)	**Found (%)***
Raw material	100.0	101.9 (0.3)
Suspension	2.5	2.7 (0.6)

*Results are expressed as mean values (n=3). RSD values in parenthesis.

IV. CONCLUSION

A LC-UV method was developed and validated for the quantification of UDCA in raw material and a

pharmaceutical formulation (suspension). This method is simple, fast, highly specific, precise, exact and robust. In conclusion, all these features make this method suitable to be used in a routine analysis of UDCA in the laboratory, for stability studies and quality control of UDCA pharmaceutical formulations.

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