Journal of Pharmacy and Pharmacology 6 (2018) 448-455
doi: 10.17265/2328-2150/2018.05.002

LC-MS/MS Method Applied to the Detection and Quantification of Ursodeoxycholic Acid Related Substances in Raw Material and Pharmaceutical Formulation

Boscolo Oriana[1], Flor Sabrina[1,2,4], Dobrecky Cecilia[1,3], Martinefski Manuela[1,2], Tripodi Valeria[1,4] and Lucangioli Silvia[1,4]

1. Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Tecnología Farmacéutica, Junín 956, CABA, Argentina

2. Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Química Analítica, Junín 956, CABA, Argentina

3. Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Farmacología, Junín 956, CABA, Argentina

4. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, CABA, Argentina

Abstract: Objective: To develop a highly sensitive LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry) method applied to the detection and quantitation of UDCA (ursodeoxycholic acid) related substances such as CA (cholic acid), DCA (deoxycholic acid), CDCA (chenodeoxycholic acid) and LCA (lithocholic acid) in raw material and pharmaceutical formulation. Methods: The method was validated for specificity, linearity, accuracy, precision, robustness. A triple quadrupole mass detector was employed, equipped with an ESI (electrospray ionization) source operated in the negative ion mode. The chromatographic system consisted of a Symmetry C18 column (150 mm × 4.6 mm, id; particle size 5 µm) and methanol-acetonitrile-ammonium acetate (pH 7.6; 10 mM) (40:40:20, v/v/v) as the mobile phase. The chromatographic conditions were 25 uL injection volume, flow rate of 0.4 mL/min and column temperature set at 35 °C. Key findings: The method requires a minimum sample amount and presents very low LOD (limits of detection) for CA (0.29 ng/mL), DCA (0.59 ng/mL), CDCA (0.13 ng/mL) and LCA (0.44 ng/mL) in comparison to LC methods coupled to different detectors like UV (ultraviolet), fluorescence and refractive index. Conclusions: The developed and validated LC-MS/MS method for the determination of UDCA and related substances in raw material and in a suspension was advantageous since it required a minimum sample amount. In turn, it could be used as a stability indicating method.

Key words: UDCA, related substances, LC, mass detector, pharmaceutical formulation.

1. Introduction

UDCA (Ursodeoxycholic acid), (3α, 7β-dihydroxy-5β-cholan-24-oic acid), also known as ursodiol, is a naturally occurring BA (bile acid). BAs are steroid compounds, hydroxyl derivatives of 5β-cholan-24 oic acid [1]. Primary BA, CA (are cholic acid) and CDCA (chenodeoxycholic acid); secondary BA such as DCA (deoxycholic acid) and LCA (lithocholic acid), all of them in 3α-position, and UDCA (3β-position) (Fig. 1). UDCA is the epimer of CDCA (3α-position), this structural modification transforms UDCA in a less hydrophobic, detergent and toxic BA [2]. Therefore, UDCA has been used as a therapeutic agent for the treatment of hepatobiliary disorders such as cholestasis, biliary dyspepsia, primary biliary cirrhosis and different cholestatic conditions [3, 4].

UDCA raw material is obtained from an ox-bile [4]. CDCA (a poorly tolerated BA), LCA and DCA cause

Corresponding author: Silvia Lucangioli, Ph.D., full professor, research fields: pharmaceutical technology.

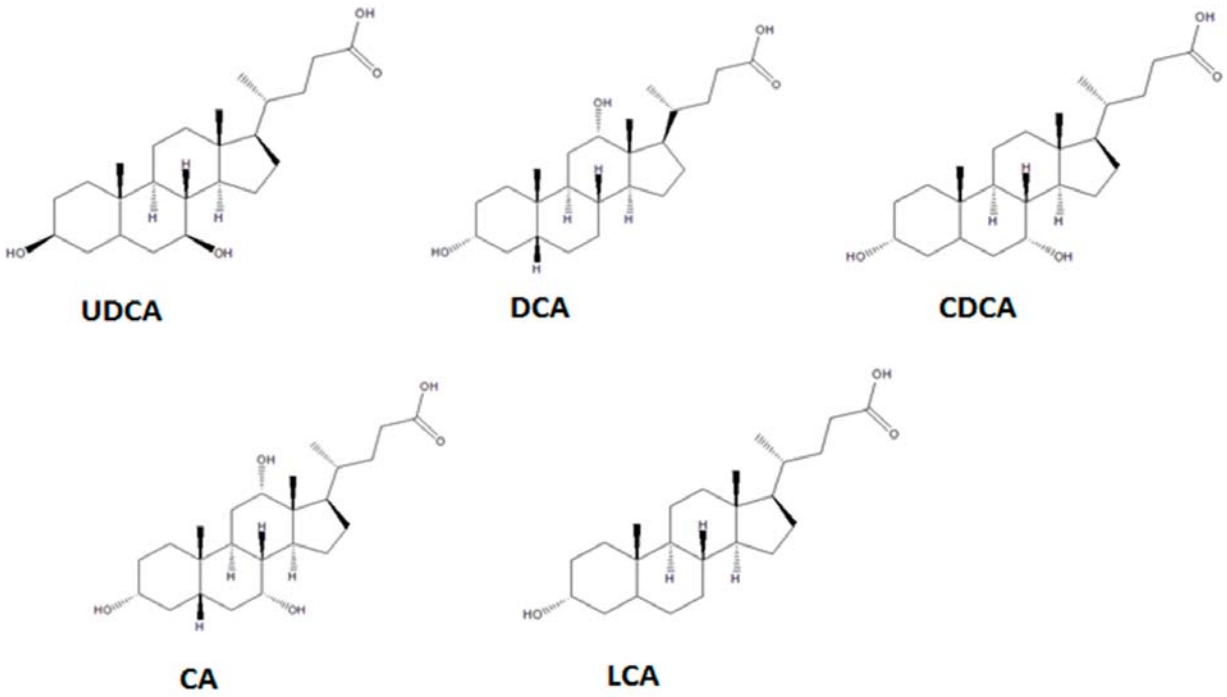

Fig. 1 Chemical structure of UDCA and related substances: DCA, CDCA, CA and LCA.

hepatotoxicity, with LCA being the most toxic, all of them can be found as potential related substances [5] along with CA. The USP(United States Pharmacopeia) UDCA official monograph proposes an HPLC (high performance liquid chromatography) method coupled to a differential refractive index detector for its analysis in raw material and a TLC (thin layer chromatography) method for the evaluation of CDCA and LCA as its related substances [6]. Moreover, the EP (European Pharmacopoeia) UDCA monograph presents a TLC method for the analysis of related substances; CA, CDCA, LCA and other related BA like DCA [7]. Due to the low absorptivity of BA, high concentration is needed for UV (ultraviolet) detection and also for TLC.

Moreover, several analytical methods applied to the evaluation of the UDCA related substances have been reported. Most of them describe HPLC coupled to various detectors: UV [5], electrochemical [8], prederivatization fluorescence [5, 9], evaporative light scattering [10] and refractive index [5, 11]. Another method such as CE (capillary electrophoresis) has also been applied [12-14]. In this sense, in a previous work, we developed CE-UV methods applied to determination of BA in pharmaceutical formulations as well as the evaluation of UDCA related substances [12, 14]. Although CE methods were suitable for the analysis of BA in pharmaceutical formulations and raw material, they require high sample concentrations and CE is not common laboratory equipment.

On the other hand, HPLC tandem mass spectrometry [15] is the methodology of choice for the analysis of BA in biological samples, especially HPLC-MS (mass spectrometry) and HPLC-MS/MS which provide high sensitivity and specificity [16].

The aim of this work was to develop and validate for the first time a highly sensitive HPLC tandem MS/MS method for the evaluation of UDCA related substances in raw material and pharmaceutical formulation as a stability indicating method. To our knowledge, there is no report in literature on the use of an LC-MS/MS method to the analysis of UDCA related substances, in raw material and pharmaceutical formulation.

2. Materials and Methods

2.1 Chemicals and Reagents

UDCA, CA, DCA, CDCA and LCA were 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 from 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 0.45 µm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and before use (with vacuum).

2.2 Equipment and Chromatographic Conditions

The LC-MS/MS analyses were performed on an Ultimate 3000 System HPLC system (Thermo Fisher Scientific, Inc, San Jose, CA, USA) coupled to TSQ Quantum AcessMax mass spectrometer (Thermo Fisher Scientific, Inc, San Jose, CA, USA). The LC was equipped with an autosampler, quaternary pump, mobile phase online degasser and a thermostatted column compartment. The chromatographic separation was carried out isocratically using a Symmetry C18 column (150 mm × 4.6 mm, id; particle size 5 µm) supplied by Waters (Milford, Massachusetts, USA). The mobile phase consisted of methanol-acetonitrile-10 mM ammonium acetate (40:40:20, v/v/v). The flow rate was set at 0.6 mL/min. The column temperature was set at 40°C. The injection volume was 20 µL and the running time was 10 minutes.

The TSQ Quantum Access Max triple quadrupole mass spectrometer was equipped with an ESI (electrospray ionization) source and was operated in the negative ion mode. The optimal values for MS parameters were: 4,500 V for spray voltage, 60 and 45 for sheath and auxiliary gas pressure, respectively, 280 °C for capillary temperature and 53 eV for collision energy. Data acquisition was performed using Xcalibur 2.1 software (Thermo Fisher Scientific, Inc, San Jose, CA, USA).

2.3. Preparation of Stock, Standard and Sample Solution

2.3.1 Preparation of Stock and Standard Solutions

Totally, 400 mg of UDCA was accurately weighed and transferred to a 100 mL volumetric flask and dissolved in methanol to give a stock solution concentration of 4 mg/mL. Stock solutions of CA, DCA, CDCA, all of 4 µg/mL and 1 µg/mL LCA, were prepared by appropriate dilution in methanol/acetonitrile (50:50). Standard solution containing UDCA (40 µg/mL), CA (0.2 µg/mL), DCA (0.1 µg/mL), CDCA (0.4 µg/mL) and LCA (0.02 µg/mL) was prepared by appropriate dilution in mobile phase.

2.3.2 Preparation of Sample Solution

2.3.2.1 UDCA raw material.

For the analysis of CA, DCA, CDCA and LCA related substances, approximately 400 mg of UDCA raw material was accurately weighed in a 100 mL volumetric flask and dissolved in methanol. A 1/10 dilution was prepared in methanol/acetonitrile (50:50). This was further diluted with mobile phase to a final concentration of 40 µg/mL.

2.3.2.2 Pharmaceutical suspension.

The UDCA suspension was shaken vigorously by hand immediately before use. A total of 16 g was transferred to a 100 mL volumetric flask and filled up with methanol, sonicated for 5 min and centrifuged at 15,000 rpm for 10 min to separate the insoluble components. A 1/10 dilution of the supernatant was prepared in methanol/acetonitrile (50:50). This was further diluted with mobile phase to a final concentration of 40 µg/mL.

2.4 Method Validation

The developed method was validated according to ICH (International Council for Harmonisation) guidelines [17]. Parameters such as specificity, linearity, LOD (limits of detection) and LOQ (limits of quantification), precision, accuracy and robustness were tested.

2.4.1 Specificity

A blank of excipients of the pharmaceutical suspension was prepared to test specificity.

2.4.2 Linearity, LOD and LOQ

Linearity was assayed at five concentration levels for each related compound within the range of 50-625 ng/mL for CA, 25-425 ng/mL for DCA, 50-800 ng/mL for CDCA and 10-115 ng/mL for LCA, where each concentration was injected by triplicate. Regression coefficients were obtained by plotting the average peak area versus concentration, using the least squares method. LOD and LOQ were determined in six replicates, as 3 and 10 times S/N, respectively. RSD (relative standard deviation) for peak area was calculated.

2.4.3 Precision

Precision was evaluated for intra-day (n = 6) and inter-day (n = 18) for CA (207 ng/mL), DCA (119 ng/ml), CDCA (422 ng/mL) and LCA (21 ng/mL) under the conditions described in preparation of stock and standard solutions. Precision was determined as RSD for peak area and retention time.

2.4.4 Accuracy

Accuracy was calculated as recovery. Samples were prepared with all excipients present in the suspension, the active pharmaceutical ingredient and subsequently supplemented with CA, DCA, CDCA and LCA at three different concentration levels (related to limit values for each related substance): 80%; 100% and 120%, three replicates of each level. The 100% level is related to the limit value of each related substance, that is: not more than 0.5% for CA, not more than 0.25% DCA, not more than 1% and not more than 0.05% LCA. The RSD for each related substances area was calculated.

2.4.5 Robustness

Robustness was evaluated for flow rate (+/- 0.05 mL/min), column temperature (+/- 2°C) and injection volume (+/- 1 μL). The effect on chromatographic parameters such as retention factor, tailing factor, RSD for CA, DCA, CDCA and LCA standard solution at 207 ng/mL, 119 ng/mL, 422 ng/mL and 21 ng/mL, respectively, were evaluated.

2.4.6 Related Substances Analysis

The developed method was applied to the analysis of related substances in raw material and pharmaceutical suspension. Sample solutions were prepared according to preparation of sample solutions. Quantification of related substances was performed under the conditions described in equipment and chromatographic conditions.

3. Results and Discussion

3.1 Method Development

The aim of this work was to develop an HPLC-MS/MS method for the detection and quantification of UDCA and related substances in raw material and suspension.

The USP UDCA monograph describes CDCA and LCA as related substances, where each related substance limit should not be more than 1.5% w/w and 0.05% w/w, respectively. At the same time, the European Pharmacopoeia refers to CA, DCA, CDCA and LCA as related substances, where each related substance limit should not be more than 0.5% w/w, 0.25% w/w, 1.0% w/w and 0.1% w/w, respectively

The chromatographic conditions were optimized to achieve good resolution, symmetric peak shapes and a short run time. The use of a Waters Symmetry C18 column was more suitable for the analysis of BA as it provided better separation, especially sharper peaks as well as less retention time shift compared with other columns. The mobile phase consisted of a mixture of methanol: acetonitrile: 10 mM ammonium acetate (40:40:20). The proportion of organic solvent allows the elution of the analytes in adequate retention time.

Buffer concentration was an important factor to optimize. Higher concentrations of ammonium acetate could improve resolution, but with lower sensitivity. Therefore, 10 mM ammonium acetate was a good choice in terms of resolution and sensitivity.

The mass detector was equipped with an ESI (electrospray ionization) source and was operated in the negative ion mode as BAs have an acidic group in their chemical structure. The operational conditions were optimized to obtain an efficient ionization (equipment and chromatographic conditions).

A mix standard solution of UDCA, CA, DCA, CDCA and LCA was presented in Fig. 2.

The parent-ion and product-ion pairs of UDCA, CA, DCA, CDCA and LCA were analyzed. The unequivocal identification of CA, DCA, CDCA and LCA was achieved using the product-ion (qualifier ion) and retention times. However, given that UDCA, DCA and CDCA have the same fragmentation pattern, the quantitation of CA, DCA, CDCA and LCA was made using the respective molecular ion (which is considered as the quantifier ion). Nevertheless, SRM (selected reaction monitoring) mode of the molecular ions with a collision energy of 5 eV, was preferred to SIM (selected ion monitoring) mode in order to avoid adducts formation and improve sensitivity (Fig. 3).

3.2 Comparison of Methods

Several LC methods coupled to different detectors applied to the evaluation of UDCA related substances have been reported. However, detection limits are higher than mass detector, for example 0.08 μg/mL and 400 μg/mL for fluorescence and UV detection, respectively. Mass spectrometry provides higher sensitivity with lower detection limits.

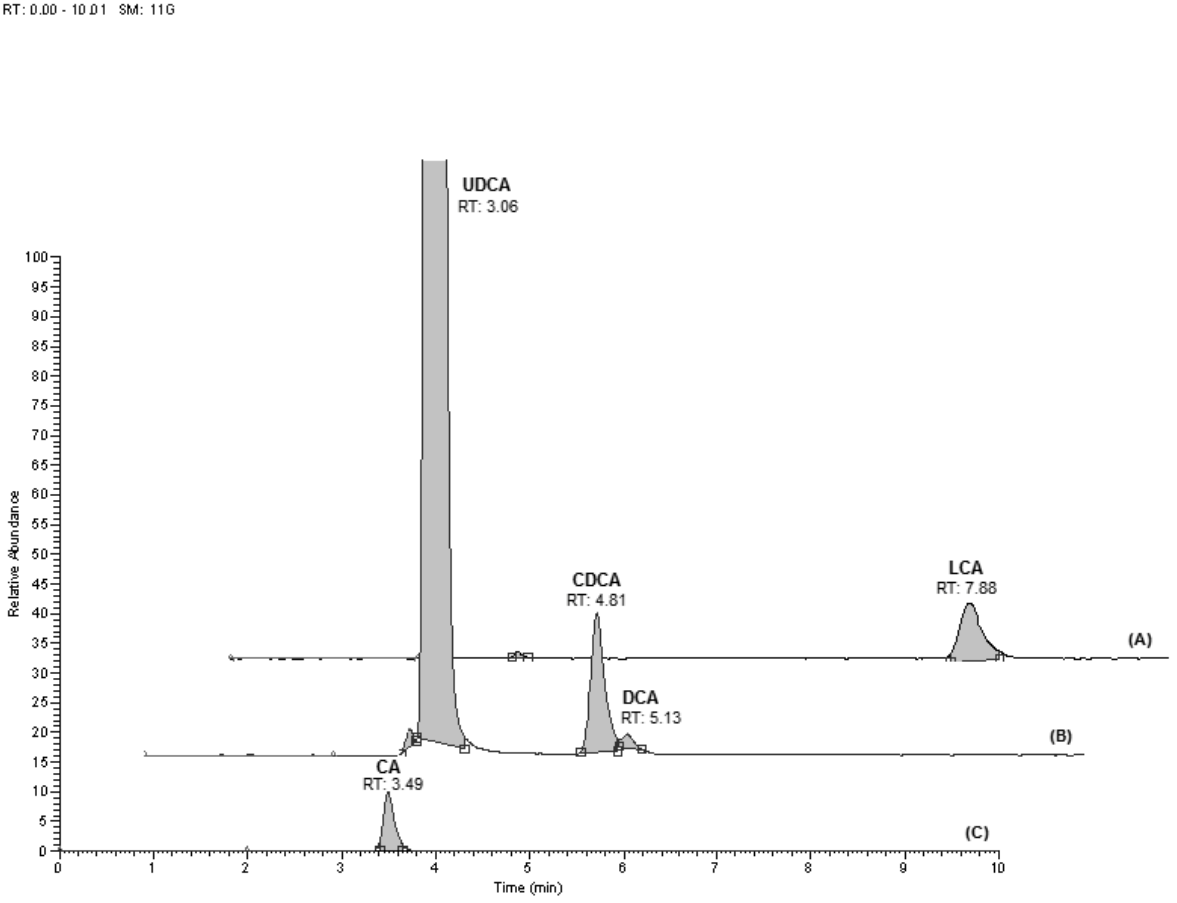

Fig. 2 Mix standard solution of UDCA, CA, DCA, CDCA and LCA. (A): LCA (21 ng/mL) scan filter 375.3 [374.8-395.8]; (B): CDCA (422 ng/mL), DCA (119 ng/mL) scan filter 391.4 [390.0-391.9] and; (C): CA (207 ng/mL) scan filter 407.4 [406.9-407.9]

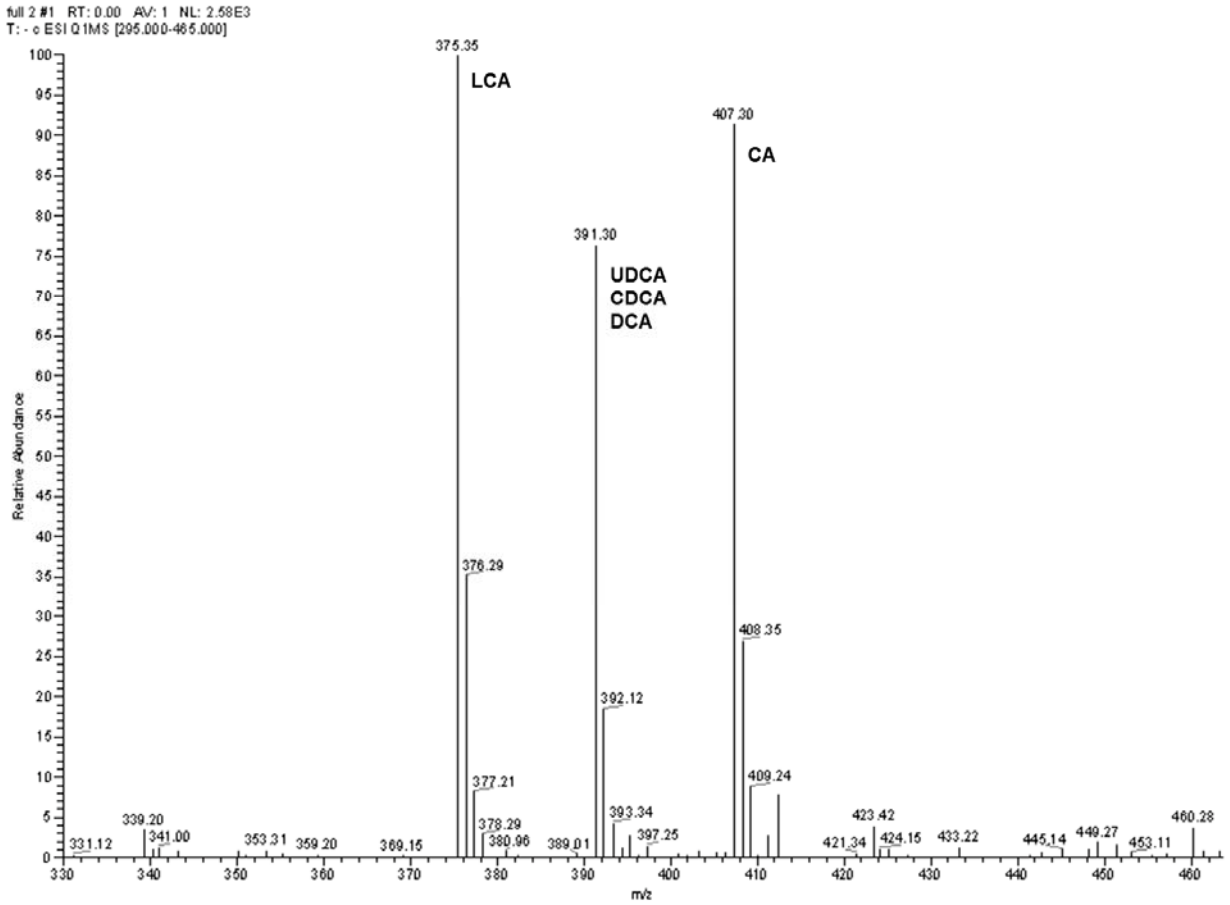

Fig. 3 Full scan mass spectra of LCA (m/z 375.35), UDCA (m/z 391.30), DCA (m/z 391.30), CDCA (m/z 391.30) and CA (m/z 407.30).

The refractive index detector is used in the USP method for UDCA raw material. In this sense, Peepliwal et al. have developed an LC method coupled to a refractive index detector using methanol instead of acetonitrile (the latter used in the USP method). However, up to 0.14% of UDCA related substances in raw material are detected with this method. Moreover, other methodologies as CE or TLC also present low sensitivity.

The use of the mass detector provides an excellent sensitivity with a minimum sample amount compared to other methods for UDCA related substances.

3.3 Method Validation

3.3.1 Specificity

This was evaluated by comparing the chromatograms of the blank of excipients of the pharmaceutical suspension with the standard solution. No peaks were observed in the chromatogram of the blank of excipients.

3.3.1 Linearity, LOD and LOQ

Linearity was evaluated in the range of 0.12-1.55%, 0.06-1.06%, 0.12-2.00% and 0.03-0.30% (% w/w respect to UDCA) for CA, DCA, CDCA and LCA, respectively. The results showed good correlation coefficients, higher than 0.9901 (Table 1). LODs for CA, DCA, CDCA and LCA were 0.0007%, 0.0020%, 0.0003% and 0.0011% (%w/w respect to UDCA), respectively. LOQs for CA, DCA, CDCA and LCA were 0.002%, 0.005%, 0.001% and 0.004% (%w/w respect to UDCA), respectively. RSD values for LOD and LOQ were lower than 2% (Table 1). The low LOD and LOQ values show the high sensitivity of the method.

Table 1 Linearity, LOD and LOQ and precision of UDCA related substances.

PARAMETERS	CA			DCA			CDCA			LCA		
Linear range (ng/mL)	50-625			25-425			50-800			10-115		
R^2	0.9976			0.9952			0.9901			0.9999		
LOD (ng/mL) Picograms on column	0.29 (0.0007)* 5.8			0.59 (0.0020)* 48			0.13 (0.0003)* 2.6			0.44 (0.0011)* 8.8		
LOQ (ng/mL) Picograms on column	0.96 (0.002)* 19.2			1.96 (0.005)* 160.2			0.45 (0.001)* 9.0			1.47 (0.004)* 29.4		
Precision (RSD)												
Intra-day (n = 6)												
Peak area	1.1			0.2			2.1			0.6		
Migration time	0.1			0.3			0.3			0.1		
Inter-day (n = 12)												
Peak area	2.0			2.5			2.3			1.2		
Migration time	0.2			0.3			0.3			0.2		
Accuracy Spiked levels	80%**	100%**	120%**	80%**	100%**	120%**	80%**	100%**	120%**	80%**	100%**	120%**
Raw material***	100.2 (0.3)	98.6 (1.0)	95.0 (0.7)	100.9 (1.1)	104.5 (1.3)	105.3 (0.6)	100.5 (1.8)	96.5 (1.3)	97.8 (1.0)	102.7 (0.8)	97.1 (0.3)	106.3 (1.6)
Suspension***	93.4 (2.6)	108.9 (0.9)	104.7 (1.0)	96.3 (1.1)	90.6 (1.2)	106.0 (0.6)	91.8 (0.8)	96.7 (1.8)	104.8 (1.3)	93.5 (1.4)	90.7 (1.8)	106.2 (0.4)

* %W/W respect to UDCA. ** respect to limit values. *** RSD values between brackets corresponding to n = 3.

Table 2 Related substances in raw material and Suspension.

	CA (%)*	DCA (%)*	CDCA (%)*		LCA (%)*	
Limit	EP 0.5%	EP < 0.25%	USP 1.5%	EP 1.0%	USP 0.05%	EP 0.1%
Raw material**	-	-	1.1 (1.9)		0.025 (1.2)	
Suspension**	-	-	0.002 (0.8)		0.0003 (1.7)	

* %W/W respect to UDCA.

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

3.3.2 Precision and Accuracy

Precision and accuracy results are shown in Table 1. The method provides good precision with RSD values lower than 0.3% for retention time; as for peak areas, RSD values were lower than 2.5%. Recovery values in raw material and suspension ranged from 95.0-106.3% to 90.6-108.9%, respectively. The results obtained for precision and accuracy studies were in good agreement with international requirements.

3.3.3 Robustness

All parameters were evaluated in terms of CA, DCA, CDCA and LCA retention factor, tailing factor and RSD for replicates of standard solutions (n = 6). In all cases, RSD values lower than 3% were obtained; this demonstrates that the proposed method is suitable for the determination of UDCA related substances.

3.3.4 Analysis of Related Substances in Raw Material and pharmaceutical Suspension

Once the validation was completed, CA, DCA, CDCA and LCA quantification in raw material and suspension was evaluated. CA and DCA have not been found in raw material and pharmaceutical formulations. The results are showed in Table 2.

4. Conclusions

An LC-MS/MS method for the quantification of CA, DCA, CDCA and LCA as related substances in UDCA raw material and pharmaceutical suspension was

developed and validated for the first time. This method is simple, fast, precise, exact and robust and specially, highly specific and sensitive allowing the use of a minimal sample amount. In conclusion, this method can be used for UDCA routine laboratory analysis and to monitor the stability and quality control in UDCA pharmaceutical formulations.

Acknowledgments

The authors wish to thank University of Buenos Aires and CONICET for the support.

References

[1] Roda, A., Gioacchini, A. M., Manetta, A. C., Cerrè, C., Montagnani, M., Fini, A., et al. 1995. "Bile Acids: Physico-Chemical Properties, Function and Activity." *J. Gastroenterol.* 27: 327-31.

[2] Lucangioli, S. E., Carducci, C. N., Tripodi, V. P., and Kenndler, E. 2001. "Retention of Bile Salts in Micellar Electrokinetic Chromatography: Relation of Capacity Factor to Octanol-Water Partition Coefficient and Critical Micellar Concentration." *J. Chromatograph. B* 765: 113-20.

[3] Hofmann, A. F. 1994. "Pharmacology of Ursodeoxycholic Acid: An Enterohepatic drug." *Scand. J. Gastroenterol.* 29: 1-15.

[4] Reardon, J., Hussaini, T., Alsahafi, M., Azalgara, V. M., Erb, S. R., Partovi, N., et al. 2016. "Ursodeoxycholic Acid in Treatment of Non-cholestatic Liver Diseases: A Systematic Review." *J Clin.Transl. Hepatol.* 4: 192-205.

[5] Roda, A., Gatti, R., Cavrini, V., Cerrè, C., and Simoni, P. 1993. "HPLC Study of the Impurities Present in Different Ursodeoxycholic Acid Preparations: Comparative Evaluation of Four Detectors." *J. Pharm. Biomed. Anal.* 11: 751-60.

[6] The United States Pharmacopeia. 2017. *Official Monographs, Ursodiol* (40th Ed.). Accessed on 03 August 2017. http://app.uspnf.com/uspnf/pub/index?usp=40&nf=35&s=1&officialOn=August%201,%202017.

[7] European Pharmacopoeia 6.0. Monographs T-Z, Ursodeoxycholic Acid. 2008. Accessed 20 April 2017: http://www.uspbpep.com/ep60/ursodeoxycholic%20acid%20%201275e.pdf.

[8] Scalia, S., Tirendi, S., Pazzi, P., and Bousquet, E 1995. "Assay of Free Bile-Acids in Pharmaceutical Preparations by HPLC with Electrochemical Detection." *Int. J. Pharm.* 115: 249-53.

[9] Nobilis, M., Pour, M., Kuneš, J., Kopecký, J., Květina, J., Svoboda, Z., et al. 2001. "High-Performance Liquid Chromatographic Determination of Ursodeoxycholic Acid after Solid Phase Extraction of Blood Serum and Detection-Oriented Derivatization." *J. Pharm. Biomed. Anal.* 24: 937-46.

[10] Rajevic, M., and Betto, P. 1998. "Assay of Ursodeoxycholic Acid and Related Impurities in Pharmaceutical Preparations by HPLC with Evaporative Light Scattering Detector." *J. Liq. Chromatograph. Relat.Technol.* 21: 2821-30.

[11] Peepliwal, A., Bonde, C. G., and Bothara, K. G. 2011. "A Validated RP-HPLC Method for Quantitative Determination of Related Impurities of Ursodeoxycholic Acid (API) by Refractive Index Detection." *J. Pharm. Biomed. Anal*. 54: 845-9.

[12] Rodríguez, V. G., Lucangioli, S. E., Fernández Otero, G. C., and Carducci, C. N. 2000. "Determination of Bile Acids in Pharmaceutical Formulations Using Micellar Electrokinetic Chromatography." *J. Pharm. Biomed. Anal.* 23: 375-81.

[13] Quaglia, M. G., Farina, A., Bossù, E., Dell'Aquila, C., and Doldo, A. 1997. "The Indirect UV Detection in the Analysis of Ursodeoxycholic Acid and Related Compounds by HPCE." *J. Pharm. Biomed. Anal.* 16: 281-5.

[14] Lucangioli, S. E., Rodriguez, V. G., Fernández Otero, G. C., and Carducci, C. N. 1998. "Determination of Related Impurities of Bile Acids in Bulk Drugs by Cyclodextrin-Modified Micellar Electrokinetic Chromatography." *J. Capillary Electrophor.* 5: 139-42.

[15] Perwaiz, S., Tuchweber, B., Mignault, D., Gilat, T., and Yousef, I. M. 2001. "Determination of Bile Acids in Biological Fluids by Liquid Chromatography-Electrospray Tandem Mass Spectrometry." *J. Lipid. Res.* 42: 114-9.

[16] Tripodi, V., Lucangioli, S. E., Scioscia, S. L., and Carducci, C. N. 2003. "Simultaneous Determination of Free and Conjugated Bile Acids in Serum by Cyclodextrin-Modified Micellar Electrokinetic Chromatography." *J. Cromatograph. B* 785: 147-55.

[17] ICH, 2005. Q2 (R1), "Validation of analytical procedures: text and methodology", ICH Harmonised Tripartite Guideline. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Chicago, USA.

[18] Accessed on April 20, 2017. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.