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High-performance liquid chromatographic mass spectrometric method for the determination of ursodeoxycholic acid and its glycine and taurine conjugates in human plasma

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Abstract

A novel sensitive high-performance liquid chromatography-electrospray mass spectrometry method has been developed for the determination of ursodeoxycholic acid (UDCA) and its glycine and taurine conjugates, glycoursodeoxycholic acid (GDCA) and tauroursodeoxycholic acid (TDCA). The procedure involved a solid phase extraction of UDCA, GDCA, TDCA and the internal standard, 23-nordeoxycholic acid from human plasma on a C18 Bond Elut cartridge. Chromatography was performed by isocratic reverse phase separation with methanol/25 mM ammonium acetate (40/60, v/v) containing 0.05% acetic acid on a C18 column with embedded polar functional group. Detection was achieved using an LC-MS/MS system. The standard curve was linear over a working range of 10–3000 ng/ml for all analytes and gave an average correlation coefficient of 0.9992 or better during validation. The absolute recovery for UDCA, GDCA, TDCA and the internal standard was 87.3, 83.7, 79.5 and 95.8%, respectively. This method is simple, sensitive and suitable for pharmacokinetics, bioequivalence or clinical studies.
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1. Introduction

Ursodeoxycholic acid (3-α,7-β-dihydroxy-5-β-cholanic acid, UDCA) is a naturally occurring bile acid found in small quantities in human plasma. UDCA is widely used for the dissolution of gallstones and used for the treatment of cholestatic liver disease such as primary biliary cirrhosis, primary sclerosing cholangitis and chronic hepatitis [1–3].

Several analytical methods have been developed and published for the determination of bile acids in biological fluids. Among the methods described in the literature, there are HPLC methods with ultraviolet, fluorescence or refractive index detection [4–10], HPLC with mass spectrometric detection [11,12] or gas chromatography with mass spectrometric detection [1,12–15]. With most of these methods, free and conjugated bile acids are not analyzed simultaneously. In some cases, the extraction procedure divides the compounds into two fractions for analysis, or only focuses on conjugated bile acids. In other cases, the sample preparation or the detection technique is different for each class of compound. Furthermore, derivatization is often required to obtain a low ng/ml detection limit.

In this paper, we present a simple method for the simultaneous determination of UDCA, glycoursodeoxycholic acid (GDCA) and tauroursodeoxycholic acid (TDCA) in human plasma. Fig. 1 represents the structures of these compounds. The method requires an HPLC with mass spectrometric detection. Mass spectrometric detection with electrospray ionization offers a detection technique capable of analyzing all analytes with high sensitivity. This detection method combined with liquid chromatography allows the simultaneous analysis of all analytes within a 5 min run time. In addition, this method involves simple solid phase extraction and gives reliable reproducibility that makes it suitable for pharmacokinetics, bioequivalence or clinical studies.

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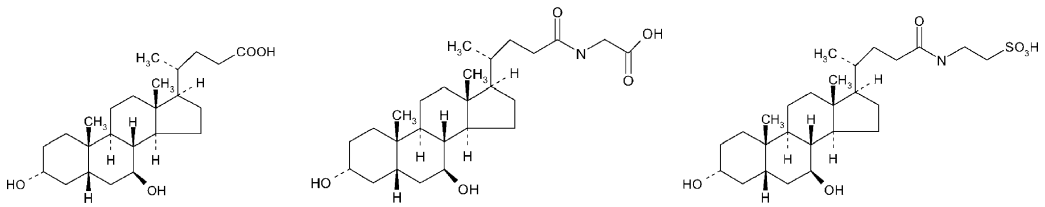

Fig. 1. Chemical structures of UDCA, GDCA and TDCA.

2. Experimental

2.1. Materials

Ursodeoxycholic acid was supplied by Pharmascience (Montreal, Canada) Glycoursodeoxycholic acid was obtained from Calbiochem (California, USA). Tauroursodeoxycholic acid was obtained from Sigma (Oakville, Canada) and 23-Nordeoxycholic acid from Steraloids. (Newport, USA). The human plasma was obtained from Biological Specialty (Colmar, PA, USA). HPLC grade water was supplied from an in house Nano-pure water purification system. Bond Elut C18 3cc/100 mg cartridges were obtained from Varian, (Mississauga, Ont., Canada). Methanol, ammonium formate and glacial acetic acid were purchased from Fisher Scientific (Nepean, Ont., Canada). Formic acid was obtained from BDH (Toronto, Ont., Canada).

2.2. Stock solutions and standards

Stock solutions of UDCA, GDCA, TDCA and 23-nordeoxycholic acid (internal standard) were prepared by mixing an appropriate amount of the drug with methanol to obtain a final concentration of 1.0 mg/ml for UDCA, GDCA and TDCA and 0.1 mg/ml for 23-nordeoxycholic acid. The stock solutions were stored at $-20 \pm 5\,^{\circ}C$. A seven point non-zero calibration standard, ranging from 10 to 3000 ng/ml was prepared by spiking the human plasma containing EDTA with an appropriate amount of the analytes. The quality control (QC) samples were prepared in a manner similar to the calibration standards and at three concentration levels (30, 1200 and 2200 ng/ml). The quality control samples were extracted with the calibration standards to verify the integrity of the method.

2.3. Sample preparation

The solid phase extraction cartridge (Bond Elut C_{18}) was conditioned with 1 ml of methanol and 1 ml of 0.05% acetic acid solution prior to the sample loading. The plasma samples were prepared by mixing 0.3 ml of plasma with 150 μl of internal standard solution that contained 1.5 mcg/ml of 23-nordeoxycholic acid and 1 ml of 0.05% acetic acid solution. The sample mixture was loaded on the conditioned cartridge and then washed with 1.0 ml of 0.05% acetic acid solution and 1 ml of methanol/water (45/55). The cartridge was then eluted with 1.5 ml of methanol. The eluent was dried under nitrogen and then reconstituted with 150 μl of mobile phase; 20 μl of reconstituted sample was injected for analysis.

2.4. Chromatography and quantitation

The chromatographic system consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA). The separation was achieved by using a 100 mm × 4.6 mm, 5 μm Prism RP column (Thermo Hypersil Keystone, Bellefonte, PA, USA) with a mobile phase consisting of methanol/25mM ammonium acetate (40/60, v/v) with 0.05% acetic acid. The mobile phase was delivered at a flow rate of 1.0 ml/min with split ratio of 1:5. The split ratio was optimized to allow an optimal evaporation of the mobile phase in order to optimize the sensitivity. Optimal evaporation of the mobile phase enhances the ion formation and increases the ruggedness of the assay by preventing contamination of the LC/MS/MS system. Under these conditions, UDCA, GDCA and TDCA eluted at 3.65, 4.25 and 4.20 min with a total run time of 5 min. No ion suppression was observed by the co-eluting peaks in the other mass transitions.

Bile acids can be found in plasma under various isomeric forms, which have the same mass and product ion. The analytes and their major endogenous isomers are chromatographically separated. Chromatographic separation is demonstrated in Fig. 2.

Mass spectra were obtained with a Sciex API-2000 mass spectrometer (Concord, Ont., Canada) equipped with a turbo ion-spray source. Ions were monitored in a negative mode. The ion transitions measured were 391.3–391.3, 448.2–74.1, 498.2–80.1 and 377.2–377.2 for UDCA, GDCA, TDCA and the internal standard, respectively. Quantitation of the analytes in human plasma was based on the peak area ratios of the analytes versus the internal standard. The analyte concentrations in human plasma were determined by standard curves that were analyzed with weighted least squares linear regression (weighting factor $1/x^2$).

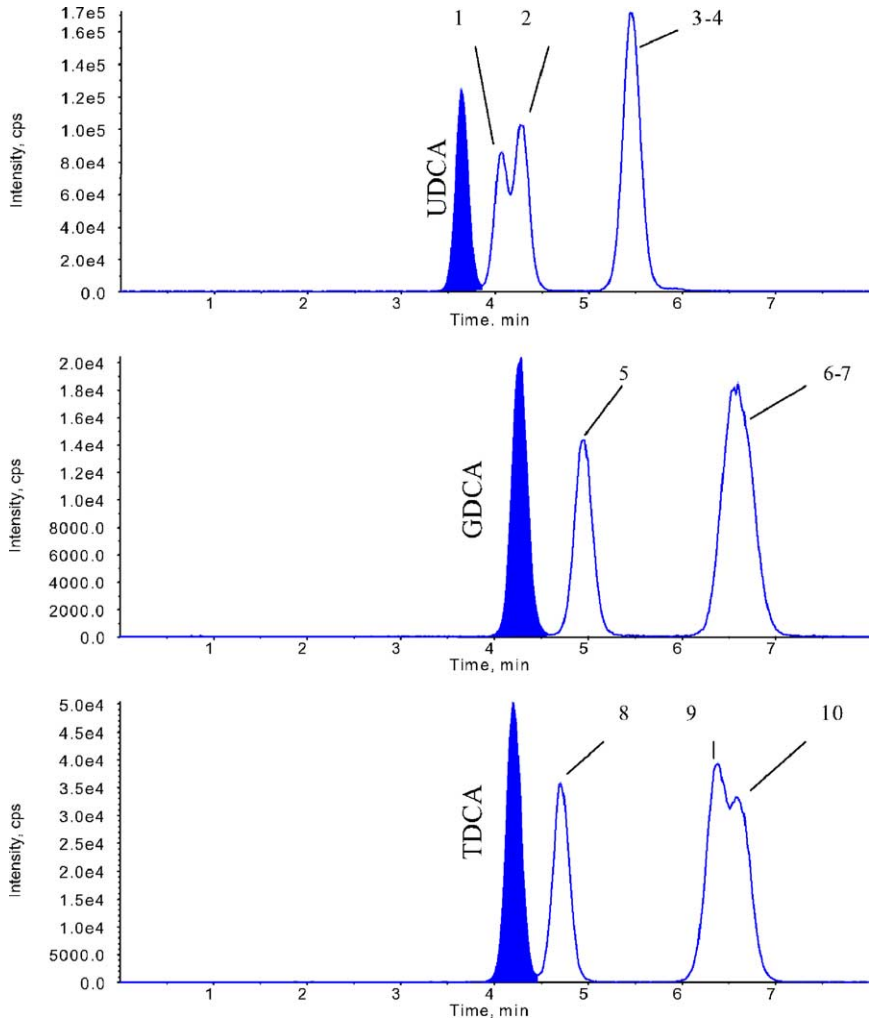

Fig. 2. Chromatographic separation between the analytes and their isomers: (1) cholanic acid 3-α, 6-β diol, (2) hyodeoxycholic acid, (3) deoxycholic acid, (4) chenodeoxycholic acid, (5) glycohyodeoxycholic acid, (6) glycodeoxycholic acid, (7) glycochenodeoxycholic acid, (8) taurohyodeoxycholic acid, (9) taurodeoxycholic acid, (10) taurochenodeoxycholic acid.

3. Results

3.1. Specificity

Human plasma was tested for interferences before spiking. No significant endogenous interference was observed at the retention time of the internal standard (<1%). However, since UDCA and its glycine and taurine conjugates are naturally occurring in human plasma, endogenous interferences were found at their respective retention time. The blanks screened ($n = 16$) contained an endogenous level between 2.5 and 94.4 ng/ml, between 5.8 and 413.9 ng/ml and between 0.0 (below limit of quantitation) and 12.3 ng/ml for UDCA, GDCA and TDCA, respectively. One of these plasma samples was then used to reconstitute the biological matrix for the calibration curve and QC sample preparation. A chromatogram of an extracted blank plasma sample as well as representative chromatogram of extracted calibration sample at the lowest limit of quantitation (LOQ), and extracted high QC sample are provided in Figs. 3–5.

Six commonly used over-the-counter drugs (OTCs) were also tested for possible ionization suppression/enhancement. The ionization suppression/enhancement observed for UDCA, GDCA, TDCA and the internal standard was below 9.2% for all drugs. The OTCs tested were the following: caffeine, acetylsalicylic acid, ibuprofen, naproxen, 4-acetamidophenol and dextromethorphan at a concentration of 5 μg/ml.

3.2. Limit of quantitation, linearity and precision

The LOQ in human plasma was 10 ng/ml for all analytes. A linear response for the peak area ratio versus concentration over a working range of 10–3000 ng/ml was observed for UDCA, GDCA and TDCA with an average correlation coefficient of 0.9992 ($n = 5$) or better.

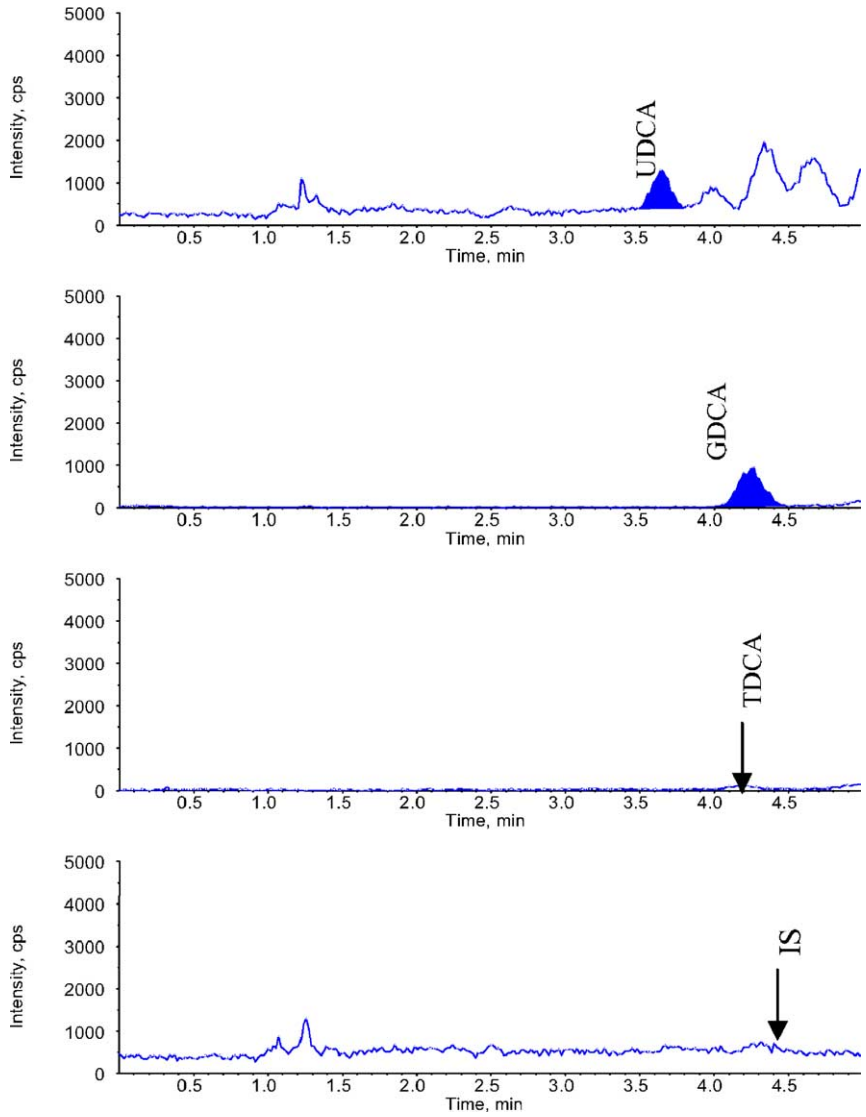

Fig. 3. Chromatograms of extracted plasma blank samples.

The inter-assay precision and accuracy were determined by analyzing five calibration curves with quality control samples at three-concentration levels on five different days. The intra-assay precision and accuracy was determined by analyzing six replicates of the LOQ samples and quality control samples at three-concentration levels, extracted on the same day. Detailed results of intra-assay and inter assay precision and accuracy are listed in Tables 1–3.

3.3. Recovery

The absolute recovery of UDCA, GDCA and TDCA were assessed by comparing the peak area of extracted QC samples in six replicates (at low, mid and high range) to reference QCs prepared in solutions at the same concentration levels. Concentrations of the six replicates were 30, 1200 and 2200 ng/ml. The overall recovery of UDCA, GDCA and TDCA were 87.3, 83.7 and 79.5%, respectively, while the recovery of the internal standard (23-nordeoxycholic acid) was 95.8%.

3.4. Stability

The stability of the extracted UDCA, GDCA, TDCA and the internal standard in the mobile phase (processed sample stability) was evaluated and the results showed that processed samples are stable at $4 \pm 2\,^{\circ}C$ for at least 66 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected 66 h after sitting in the autosampler at $4 \pm 2\,^{\circ}C$. Evaluation was based on back-calculated concentrations.

The human plasma samples containing UDCA, GDCA and TDCA were also evaluated for stability following freeze

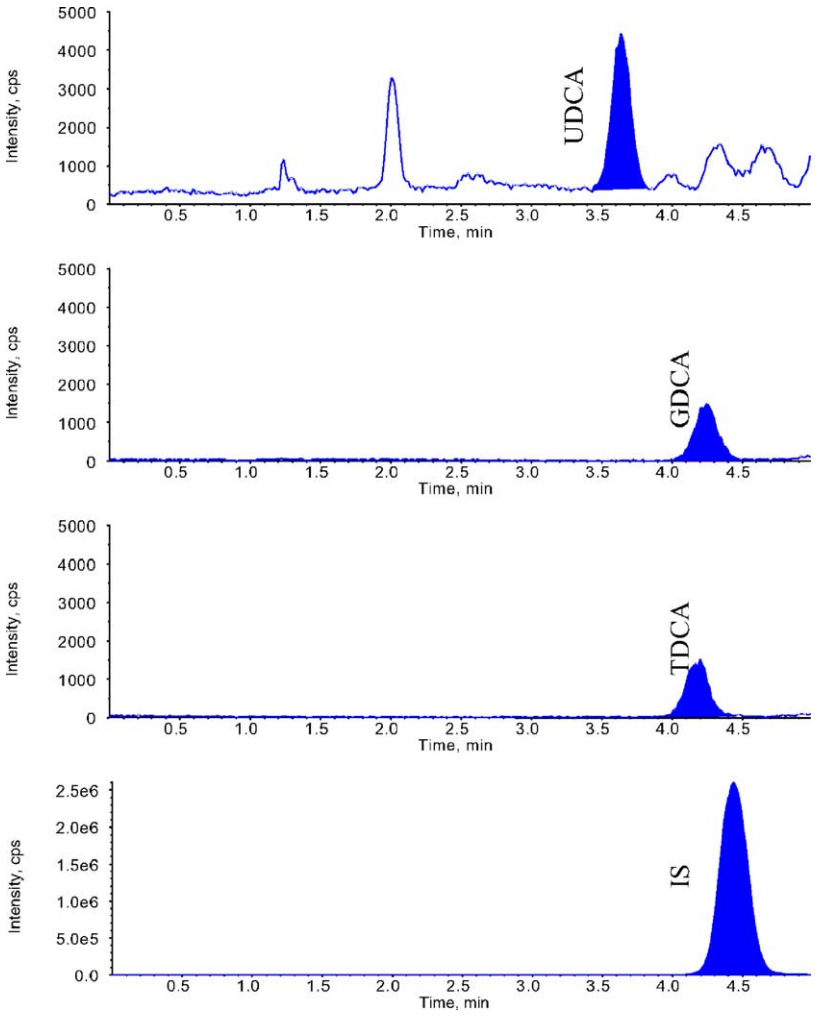

Fig. 4. Chromatograms of extracted lowest limit of quantification sample (LOQ).

and thaw. The freeze–thaw stability evaluation was conducted by comparing the back-calculated concentrations of the stability samples which had been frozen and thawed three times with the plasma samples thawed only once. UDCA, GDCA and TDCA were stable for at least three freeze–thaw cycles.

The stability of spiked human plasma samples after 105 days of storage at $-20\pm5\,^{\circ}C$ was evaluated as well. The stability evaluation involved an analysis of the low-, mid- and high-quality control samples that were stored at $-20\pm5\,^{\circ}C$ for at least 105 days, together with a freshly spiked calibration standard and quality control samples. The analysis was

Table 1
Precision and accuracy data of UDCA quality control samples

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E.[a] (%)	C.V.[b] (%)	*n*
Inter-assay				
30	28.9	−5.9	3.5	18
1200	1223	−0.4	2.9	18
2200	2224	−1.3	2.3	18
Intra-assay				
10	9.8	−3.8	4.7	6
30	28.5	−7.1	4.1	6
1200	1223	−0.4	1.8	6
2200	2219	−1.5	1.6	6

[a] R.E.: relative error.
[b] C.V.: coefficient of variation.

Table 2
Precision and accuracy data of GDCA quality control samples

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E.[a] (%)	C.V.[b] (%)	*n*
Inter-assay				
30	27.9	−6.9	2.7	18
1200	1147	−4.3	4.5	18
2200	2092	−4.9	3.6	18
Intra-assay				
10	9.1	−9.5	4.7	6
30	28.1	−6.3	4.1	6
1200	1118	−6.8	1.8	6
2200	2049	−6.9	1.6	6

[a] R.E.: relative error.
[b] C.V.: coefficient of variation.

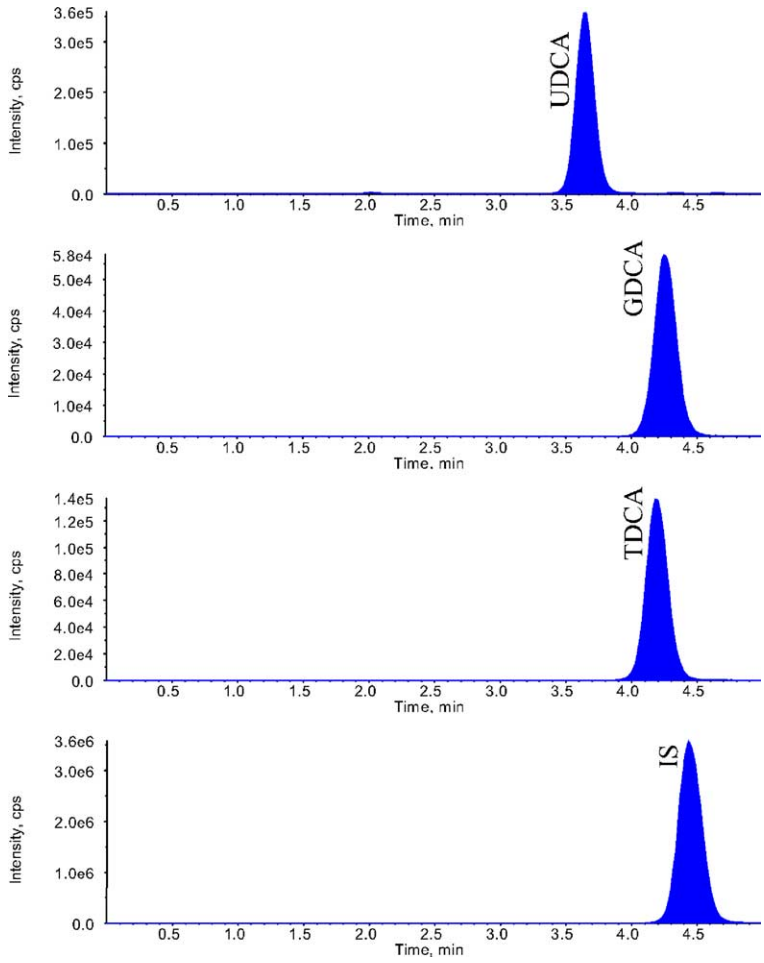

Fig. 5. Chromatograms of extracted plasma highest QC sample.

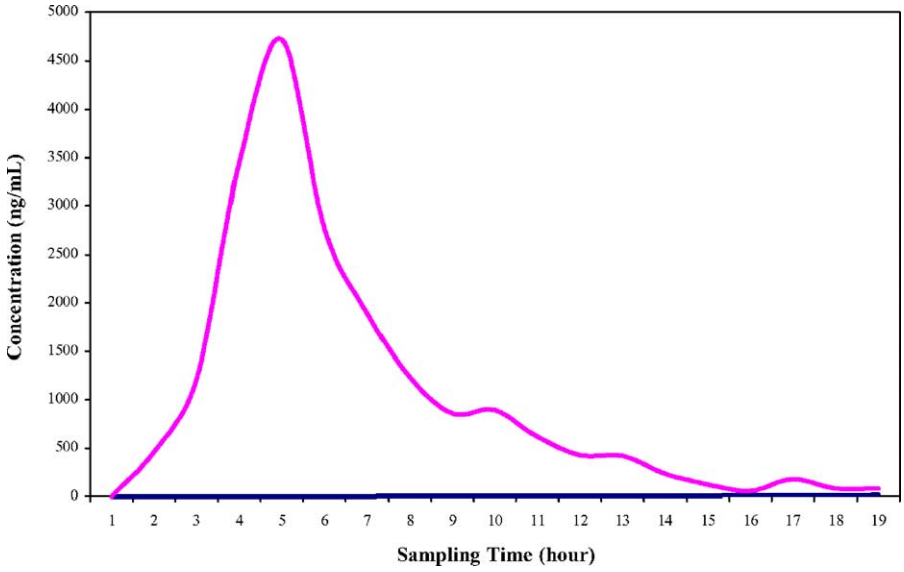

Fig. 6. Example of plasma concentration vs. sampling time profile in human subject.

Table 3
Precision and accuracy data of TDCA quality control samples

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E.[a] (%)	C.V.[b] (%)	n
Inter-assay				
30	27.8	−7.2	2.5	18
1200	1124	−6.3	2.3	18
2200	2056	−6.5	1.4	18
Intra-assay				
10	10.1	0.5	6.0	6
30	28.2	−6.1	2.2	6
1200	1127	−6.0	1.9	6
2200	2062	−6.2	1.4	6

[a] R.E.: relative error.
[b] C.V.: coefficient of variation.

performed on the same day. The back calculated values for the stability were between −5.5 and 2.5, −3.6 and 5.8, and −7.8 and −1.5% for UDCA, GDCA and TDCA, respectively, which demonstrates the stability of these substances in human plasma for at least 105 days at $-20 \pm 5\,^{\circ}C$.

3.5. Application

The method has been applied to a bio-availability study with great success. Fig. 6 shows an example of plasma concentration of ursodil versus sampling time profile in human plasma samples.

4. Discussion

In this study, mass spectrometry detection combined with liquid chromatography was used to perform the analysis of UDCA and its glycine and taurine conjugates. Mass spectrometry is a sensitive and robust mode of detection. The limit of quantitation was 10 ng/ml using 0.3 ml of plasma. The limit of quantitation could be lowered if necessary using a larger plasma volume and by using a smaller inner diameter chromatography column to avoid the 1/5 split ratio to the mass spectrometer. Other than the sensitivity, the advantage of mass spectrometry over other techniques is the ability to analyze the free and conjugated compounds simultaneously without derivatization.

UDCA and the internal standard (23-nordeoxycholic acid) do not fragment in the collision cell (Q2) to give product ions with sufficient signal to noise. When the collision energy increases, the molecular ion signal decreases to form high background in the lower mass range without producing any specific product ion. Therefore, the mass transition used were the same for the first (Q1) and the third (Q3) quadrupoles without compromising signal to noise ratio. Using transitions with the same mass for UDCA and the internal standard allowed us to analyze the taurine and glycine conjugates which produce a strong product ion simultaneously in the same experiment. To accomplish this, a specific collision energy was applied to each compound. A mild collision energy was used for the UDCA and the internal standard, and a stronger collision energy was used for the taurine and glycine conjugates.

To obtain an accurate concentration determination of plasma samples, the endogenous concentration of each analyte in the plasma used to prepare the calibration standards and QC samples must be subtracted from the calibration standards and the QC sample concentrations. This was done by subtracting the average of the peak area ratio of a zero standard, from the peak area ratio of calibration standards and QC samples.

The solid phase extraction allowed a simple efficient extraction of all analytes and the internal standard. The detector response was linear over the validated range. The results obtained from this method in terms of precision and accuracy for the calibration samples and the quality control samples demonstrates the reproducibility of the assay (Tables 1–3).

5. Conclusion

A simple, sensitive and accurate high-performance liquid chromatography mass spectrometric method for the determination of ursodeoxycholic acid and its glycine and taurine conjugates in human plasma is described. This method simplifies the analysis of bile acids in human plasma. With modifications of the chromatographic conditions this method may be applicable to other bile acids. It is a robust method suitable for routine analysis involving a large number of samples. This method has been successfully applied to a bio-equivalence study.

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