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Journal of Chromatography B, 860 (2007) 10–17

JOURNAL OF CHROMATOGRAPHY B

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High-performance liquid chromatography–tandem mass spectrometry for the analysis of bile acid profiles in serum of women with intrahepatic cholestasis of pregnancy

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Received 21 May 2007; accepted 14 September 2007
Available online 2 October 2007

Abstract

A simple, sensitive, and specific high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for the analysis of the bile acid profile has been developed. Fifteen bile acids, including free and conjugated bile acids, were separated and detected by HPLC–MS/MS. The MS detection was performed by electrospray ionization (ESI) in negative ion mode. Quantification was achieved in multiple reaction monitoring (MRM) mode with external standard curve methods. Total analysis time was 15 min for one sample including re-equilibration time of the column. The assay was linear in the range 0.02–100.0 μmol/L with correlation coefficients of standard curves for all bile acids better than 0.999. The detection limits ranged from 0.001 to 0.006 μmol/L for different bile acids. The precisions for each bile acid were CVs < 3.8% for within-day and CVs < 6.1% for between-day. The average recoveries for all bile acids studied were in the range of 86–110.0%. The developed method was applied to the analysis of clinic samples consisting of 53 women with healthy pregnancies and 43 women with intrahepatic cholestasis of pregnancy (ICP). The results revealed that the bile acid profile was markedly different between women with ICP and women with healthy pregnancies.
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Keywords: Bile acid profile; HPLC–MS/MS; Serum; External standard calibration

1. Introduction

Bile acids are a group of steroidal acids with a carboxyl group located in the side chain. Bile acids, the major products of cholesterol catabolism, are formed in the liver, conjugated mainly with glycine and taurine and secreted into the bile. Bile acids serve many important physiological functions, including cholesterol homeostasis, lipid absorption, and generation of bile flow which helps the excretion and recirculation of drugs, vitamins, and endogenous and exogenous toxins [1]. In most mammals, the common bile acids are derivatives of 5β-cholan-24-oic acids. The major metabolites in humans are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA), occurring either in the free acid form or in the conjugation form with glycine and taurine at C-24 (Fig. 1).

In hepatobiliary and intestinal diseases, the hepatic synthesis and clearance of bile acids and their intestinal absorption become abnormal, which disturb both cholesterol synthesis and its metabolism, affecting the concentration and profile of bile acids in various pool compartments (serum, liver, gallbladder, urine, and feces) [2]. Intrahepatic cholestasis of pregnancy (ICP) is a specific disease appearing during pregnancy and is usually associated with abnormal liver functions. While the cause of the disease is unknown, genetic and hormonal factors are clearly associated with ICP [3]. Although ICP is a benign disease in mothers, and the symptoms usually disappear at delivery of the babies, it is associated with increased rate of fetal distress, premature deliveries and perinatal mortality. The initial diagnosis of ICP is based on the pruritus in the absence of a rash and is supported by some laboratory tests. Elevated total serum bile acids value has been reported as an indicative parameter for ICP, but other disease may also present an elevated total serum bile acids [4]. Therefore, analysis of bile acid profiles will help to distinct ICP from other diseases associated with abnormal liver functions such as asymptomatic hypercholanaemia of pregnancy [5].

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doi:10.1016/j.jchromb.2007.09.031

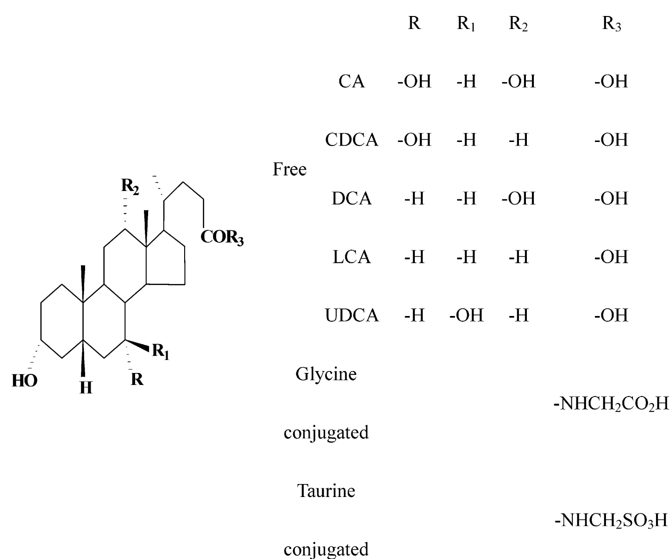

Fig. 1. Structure of bile acids.

Because of the large number of naturally occurring bile acids and their metabolites; the broad differences in physicochemical properties of bile acids such as lipophilicity and polarity; the small structural differences between individual components; and the very low concentrations in complex biological fluids, a comprehensive analysis of bile acid profile requires the use of sophisticated chromatographic techniques [6,7].

Gas chromatography–mass spectrometry (GC–MS) is a very sensitive and specific method for the detection and the quantitation of bile acids. Its major disadvantage, however, is the time-consuming derivatization prior to analysis [8]. Several HPLC methods have been described that use either ultraviolet (UV) [9] or fluorescence detection [8]. However, the shortcoming of HPLC with UV is unreliability with regard to selectivity. HPLC with fluorescence detection is widely employed for identifying and quantitating many bile acids, but use of this technique entails a series of laborious pre-analytical steps, including preliminary separation of bile acids by class, hydrolysis and derivatization, which limit its analytical performance. The coupling of HPLC to MS with atmospheric pressure ionization is very attractive due to its ability to analyze bile acids directly. A second stage of mass analysis, tandem MS (MS/MS), further enhances specificity and provides an improved signal-to-noise ratio compared with single-stage MS [10].

In this study, we have developed an HPLC–MS/MS method suitable for the quantitative analysis of the bile acid profile in serum including free and conjugated bile acids. Its application to the analysis of 15 bile acids from ICP women revealed a remarkable alteration in bile acid profile compared to controls.

2. Experiments

2.1. Materials and reagents

Methanol, acetonitrile and formic acid were of HPLC grade and purchased from Tedia (Fairfield, OH, USA). The following bile acid standards: CA, CDCA, DCA, LCA, UDCA, hyodeoxycholic acid (HDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), tauroursodeoxycholic acid (TUDCA), taurolithocholic acid (TLCA) and ammonium acetate were purchased from Sigma Chemical (St. Louis, MO, USA). HPLC grade water was obtained from Millipore pure water purification system (Millipore, USA).

Serum of women with healthy pregnancies and women with ICP was obtained from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). All serum samples were stored at −20 °C until analyzed. The sample collection and use for this study have been approved by the ethic committees of the First Affiliated Hospital of Chongqing Medical University.

2.2. Calibration standards preparation

The stock solutions of bile acids were prepared separately in methanol at the concentration of 10 mmol/L, and the stock solutions were stored at −20 °C.

A pooled serum sample was used as a biological matrix. Seven-point calibration standard solutions ranging from 0.02 to 100 μmol/L were prepared by adding appropriate amounts of each bile acid stock solution into the pooled serum. The standards were prepared in polypropylene tubes and stored at −20 °C until analyzed.

2.3. Sample preparation

The sample preparation method was based on a published method with modification [11]. Serum samples (300 μL) were mixed with 1 mL of 0.05% formic acid. The sample mixture was loaded on to reversed-phase extraction cartridges (Chromabond C18 ec, 3 mL/200 mg, MACHEREY-NAGEL GmbH & Co., KG, Düren, Germany) previously conditioned with 1 mL of methanol and 1 mL of 0.05% formic acid. The cartridges were washed with 1 mL of 0.05% formic acid and 1 mL of 45% methanol. The bile acids were eluted out from the cartridge with 1.5 mL of methanol and the eluent was dried at 60 °C under a nitrogen stream. The residue was dissolved in 100 μL of mobile phase (20% methanol, 0.01% formic acid) with ultrasonication for 2 min. Twenty microliters of samples were injected using full loop injection method (5 μL loop) to ensure that 5 μL of each sample was introduced into the HPLC–MS system.

2.4. HPLC–MS/MS analysis

For LC–MS/MS analysis, chromatographic separation was carried out on a Perkin-Elmer (Norwalk, CT, USA) HPLC. The analytical column was a C18 Waters Symmetry column (150 mm × 3.9 mm i.d. 5 μm), kept at 25 °C. The flow rate was 1 mL/min with a 1:4 post-column spilt. Chromatographic separation was achieved with gradient elution. The mobile phase A was water, and B was methanol, both contained 0.01% formic acid and ammonium acetate at 5 mmol/L. The gradient program

Table 1
Optimized parameters for MRM analysis and detection limits of the different bile acid studied

Bile acid	$[M–H]^-$ m/z	MRM	CE (V)	Retention time (min)	LOD (μmol/L)	LOQ (μmol/L)
CA	407.4	407.4 → 407.4	8	4.91	0.001	0.003
CDCA	391.3	391.3 → 391.3	8	6.79	0.002	0.006
DCA	391.3	391.3 → 391.3	8	7.04	0.002	0.006
LCA	375.3	375.3 → 375.3	8	8.76	0.002	0.006
UDCA	391.3	391.3 → 391.3	8	3.83	0.003	0.009
HDCA	391.3	391.3 → 391.3	8	4.49	0.003	0.009
GCA	464.4	464.4 → 464.4	70	2.97	0.005	0.015
GCDCA	448.3	448.3 → 74	70	4.19	0.004	0.012
GDCA	448.3	448.3 → 74	70	4.60	0.004	0.012
GLCA	432.3	432.4 → 74	65	6.04	0.005	0.015
TCA	514.4	514.4 → 80	120	2.74	0.006	0.018
TCDCA	498.4	498.4 → 80	120	3.86	0.005	0.015
TDCA	498.4	498.4 → 80	120	4.27	0.006	0.018
TLCA	482.4	482.4 → 80	120	5.65	0.003	0.009
TUDCA	498.4	498.4 → 80	120	2.10	0.003	0.009

started at 80% B and increased to 97% B in 9 min, then decreased to 80% B in 1 min and kept at 80% B for 5 min.

HPLC–MS spectra were obtained with an API 2000 mass spectrometer (Applied Biosystems-SCIEX, Concord, Ontario, Canada) equipped with a turbo ion-spray source. ESI was performed in negative ion mode with nitrogen as nebulize gas, curtain gas and heater gas, set at 30, 25 and 30 (respective arbitrary values), respectively. The ion source temperature was set at 200 °C. The ion spray voltage, focusing potential and entrance potential were set at 4200, 300 and 10 V, respectively. The MS/MS detection was operated at unit resolution in MRM mode. The pause time was set at 5 ms. Data were acquired with Analyst software, Version 1.4.1.

Standard curve was established by plotting the peak area of respective bile acid versus the concentration with correction for endogenous bile acids in the pooled sample by subtracting the peak area for each bile acid in the pooled sample.

3. Results and discussion

The main objective of this work was to develop a method to analyze bile acid profiles suitable for clinic applications. Fifteen bile acids including free, taurine and glycine-conjugated bile acids were selected for this study (Table 1) because these bile acids are the most common ones in human serum.

3.1. Sample preparation

For analysis of serum, sample preparation usually involves removal of proteins by precipitation using organic solvents followed by solid phase extraction (SPE) [12]. However, we found such a method gave poor reproducibility in our study. One explanation could be that bile acids were “co-precipitated” with proteins. Many factors such as speed of adding the organic solvent and vortex speed may affect the process of protein precipitation which may affect the recoveries of bile acids. Furthermore, the evaporation of the supernatant after protein precipitation to dryness and dissolve of the residue may also contribute to variation of the recovery of the bile acids, because the dissolve of dried residue would rarely be complete. In our method, the serum samples diluted in acidic solutions were directly applied to the solid phase extraction cartridge therefore avoided the potential problems associated with protein precipitation and the dissolve of dried residue. Acidification of the samples prompted the absorption of bile acids on the reversed-phase sorbent. After the first wash with 0.05% formic acid which removed the most of salts and polar compounds, less polar endogenous compounds were further removed by the second wash step with 1 mL of 45% methanol. After two washes, the bile acids were eluted with methanol. The recoveries and reproducibility of each bile acid were improved and were satisfactory (Table 2).

3.2. HPLC–MS/MS optimization

Bile acids have been commonly detected by ESI–MS in negative ion mode taking advantage of the presence of the carboxyl acid group [13]. For optimization of MS parameters each bile acid standard dissolved in mobile phase was analyzed by MS using direct infusion method. The parameters – such as declustering potential, collision cell entrance potential, collision cell exit potential and temperature in the ion source – were optimized in order to obtain the maximum response for deprotonated ions of bile acids. All bile acids included in this study generated predominately deprotonated ions, even in the presence of formic acid in the mobile phase. Shown in Fig. 2 are ESI-MS spectra of LCA, GLCA and TLCA at concentration of 5 μmol/L.

Then CID experiments were carried out for each bile acid to establish optimal conditions for MRM experiments, e.g. collision energy. The most suitable collision energy was determined by observing the maximum response obtained for a characteristic fragment ion, e.g. 80 m/z for taurine-conjugated bile acid peak, 74 m/z for glycine-conjugated bile acid. All free bile acids either did not generate any characteristic fragment ion (CE 8–70 V) or fragment too much so that no signal could be detected when CE was above 70 V. The product ion mass spectra

Table 2
Precision, accuracy and recoveries data of the different bile acids ($n = 5$)

Bile acids	Within-day ($\bar{X}$ ± S.D., μmol/L)	CVs (%)	Between-day $\bar{X}$ ± S.D., μmol/L)	CVs (%)	Spiked concentration (μmol/L)	Recovery (%)	CVs (%)	RE (%)
CA	0.96 ± 0.01	1.0	0.95 ± 0.02	2.1	0.50	104.0	3.1	2.1
	9.87 ± 0.28	2.8	9.85 ± 0.30	3.0	10.0	92.4	2.5	−5.6
CDCA	0.93 ± 0.01	1.1	0.93 ± 0.02	2.1	0.50	96.0	4.3	−2.1
	9.97 ± 0.13	1.3	10.02 ± 0.22	2.2	10.0	95.2	1.9	−4.6
DCA	1.08 ± 0.03	2.8	1.08 ± 0.03	2.8	0.50	90.0	3.6	−4.3
	10.40 ± 0.24	2.3	10.29 ± 0.44	4.3	10.0	93.2	1.1	−6.4
LCA	0.53 ± 0.02	3.8	0.52 ± 0.02	3.8	0.50	90.0	4.4	−8.8
	9.53 ± 0.27	2.8	9.53 ± 0.17	1.8	10.0	94.8	3.6	−5.2
UDCA	0.80 ± 0.02	2.5	0.78 ± 0.04	5.1	0.50	92.0	3.8	−4.9
	9.94 ± 0.22	2.2	9.97 ± 0.19	1.9	10.0	96.1	2.7	−3.8
HDCA	0.60 ± 0.01	1.7	0.60 ± 0.02	3.3	0.50	98.0	1.6	−1.6
	9.79 ± 0.12	1.2	9.72 ± 0.31	3.2	10.0	96.7	1.5	−3.3
GCA	1.44 ± 0.04	2.8	1.43 ± 0.03	2.1	0.50	88.0	6.2	−4.0
	11.55 ± 0.23	2.0	11.46 ± 0.38	3.3	10.0	105.7	0.9	5.2
GCDCA	2.30 ± 0.07	3.0	2.31 ± 0.06	2.6	0.50	110.0	2.2	2.2
	11.29 ± 0.19	1.7	11.14 ± 0.47	4.2	10.0	95.4	1.5	−3.9
GDCA	1.80 ± 0.05	2.8	1.79 ± 0.07	3.9	0.50	110.0	3.3	2.8
	11.04 ± 0.32	2.9	11.03 ± 0.60	5.4	10.0	91.7	2.4	−7.4
GLCA	0.56 ± 0.01	1.8	0.56 ± 0.01	1.8	0.50	92.0	3.8	−6.7
	9.16 ± 0.11	1.2	9.19 ± 0.33	3.6	10.0	90.5	2.6	−9.4
TCA	1.16 ± 0.02	1.7	1.14 ± 0.07	6.1	0.50	92.0	2.6	−3.4
	10.50 ± 0.17	1.6	10.46 ± 0.24	2.3	10.0	98.0	1.3	−1.9
TCDCA	1.45 ± 0.02	1.4	1.42 ± 0.07	4.9	0.50	86.0	6.9	−4.6
	10.51 ± 0.07	0.7	10.44 ± 0.22	2.1	10.0	94.9	2.1	−4.6
TDCA	0.85 ± 0.02	2.3	0.85 ± 0.02	2.4	0.50	102.0	1.2	1.2
	9.95 ± 0.18	1.8	9.88 ± 0.30	3.0	10.0	95.8	2.0	−4.0
TLCA	0.52 ± 0.01	1.9	0.52 ± 0.01	1.9	0.50	94.0	5.7	−5.4
	9.16 ± 0.12	1.3	9.16 ± 0.12	1.3	10.0	91.0	2.8	−8.9
TUDCA	0.53 ± 0.01	1.9	0.53 ± 0.02	3.8	0.50	92.0	3.8	−7.1
	9.36 ± 0.23	2.5	9.37 ± 0.21	2.2	10.0	93.0	1.3	−7.0

of LCA, GLCA and TLCA were presented in Fig. 2(D–F), where $[M–H]^-$ ion of each analyte was selected as the precursor. The most abundant fragment ion at *m/z* 74 and 80 were chosen for MRM acquisition for glycine and taurine-conjugated bile acid, respectively; while the unfragmented precursor ion was chosen for free bile acids. All MRM sets were summarized in Table 1. For free bile acids, MRM were set to monitoring the same precursor ions and product ions because of the lack of intense product ions. This was a common problem in LC–MS/MS analysis of free bile acids [10,11,14] and would reduce the specificity of the method. However, compared to SIM mode, it still holds advantages in term of specificity. Potential endogenous interference compounds which have the same *m/z* value would very like fragment to some extent in the collision cell under the condition used therefore the survived $[M–H]^-$ ion, if any, would be less intense compared to SIM where no CID was applied.

Among the bile acids studied, CDCA, DCA, UDCA and HDCA, were structure isomers. They give deprotonated ions at the same *m/z* and their MS/MS spectra are also similar to each other. Therefore, the detection of above four bile acids has to rely on a sufficient chromatographic separation.

We have tested different mobile phase and different additives for optimal separation of these bile acids. Methanol as a mobile phase was found to be superior to acetonitrile in terms of sharpness of bile acid peaks, potentially by contributing to the solubility of the bile acids. Acidic mobile phase normally gives better separation but suppresses the ESI signal in negative ion mode, therefore a compromise has to be made to achieve sufficient separation and sensitivity simultaneously. We found 5 mmol/L of ammonium acetate and 0.01% formic acid give the best results. In order to reduce analysis time, different gradient program were tested in order to reduce analysis time while keeping sufficient separation. A gradient starting at 80% of methanol allowed all bile acid elute within 10 min while no interference was observed. We achieved the simultaneous separation of 15 free, glycine- and taurine-conjugated bile acid in less than 15 min including the re-equilibration time. Representative chro-

Fig. 2. Negative ion mass spectra of LCA (A), GLCA (B), TLCA (C), and MS/MS spectra of LCA (D), GLCA (E) and TLCA (F).

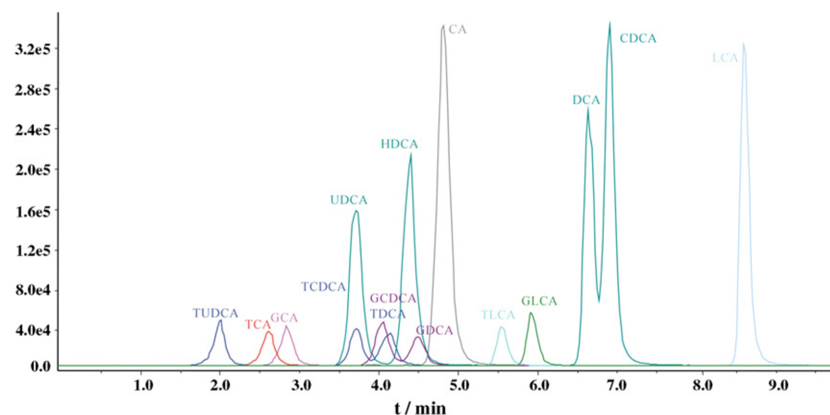

Fig. 3. Chromatograms of 15 bile acids, each at concentration of 5 μmol/L.

matograms were shown in Fig. 3. Four structure isomers: CDCA, DCA, UDCA and HDCA were separated from each other. Some bile acids were not totally separated but fortunately they could be differentiated with different MRM. Complete separation of two isomeric bile acid, DCA and CDCA, was not achieved by changing gradient profiles. Nonetheless, the reproducibility of these two bile acids was in satisfactory.

3.3. Detection limit, linearity, recoveries and precision

Because these bile acids are endogenous in human serum, their LOD and LOQ were calculated using the following formula: LOD = $3.3\,\sigma/S$; LOQ = $10\,\sigma/S$, where σ = the standard deviation of the peak area of each bile acid in pooled serum sample, S = the slope of the calibration curve [15]. Detection limits for each studied bile acid were summarized in Table 1. The linearity of response was tested by analyzing standard samples. The calibration curves are linear over the range 0.02–100.0 μmol/L.

The recovery of bile acids was determined by adding known amounts of standard solution of each bile acid to aliquots of serum sample and measuring the subsequent concentrations increase against aqueous standard calibration. The results given in Table 2 show good quantitative recoveries for all bile acids. The mean recovery of the extraction procedures was in range of 86.0–110%.

The precision and accuracy were investigated on two conditions: within run (intra-run) and between run (inter-run). The precision and accuracy were studied by repeat analysis of spiked pooled serum at two concentrations. The intra-assay precision and accuracy were determined by analyzing five replicates of the spiked serum sample at two concentration levels on the same day. The inter-assay precision and accuracy were measured by analyzing the spiked serum sample on five different days. The results given in Table 2 show that the reproducibility of the method for all bile acids studied were satisfactory.

Quantification using an internal standard is the most common method in bioanalysis. However, for a multiple analytes analysis as in our study, it is very difficult to find an internal standard suitable for all the analytes that may possess very different chemical properties and cross a wide range in concentrations. Using of multiple deuterated standards would increase the cost of the method dramatically making it unfavorable in clinic applications. The advantages of using internal standards include correction of variations in recovery especially in sample preparation, variation in injection, ionization efficiency and MS instrument variations. Among these variables, those associated with sample preparation procedure are the most difficult ones to be controlled. With modern LC–MS instrumentation, the factors influence the ionization and detection response of MS such as flow rate, sampling rate in ES process, and detector sensitivity can be controlled with satisfactory. Sample injection reproducibility also plays a role in reproducibility of quantitation analysis. If experiments have been set so that the variations determining the recovery, injection of samples, ionization efficiency and MS instrument sensitivity were minimized, an external standard curve method can also give a good

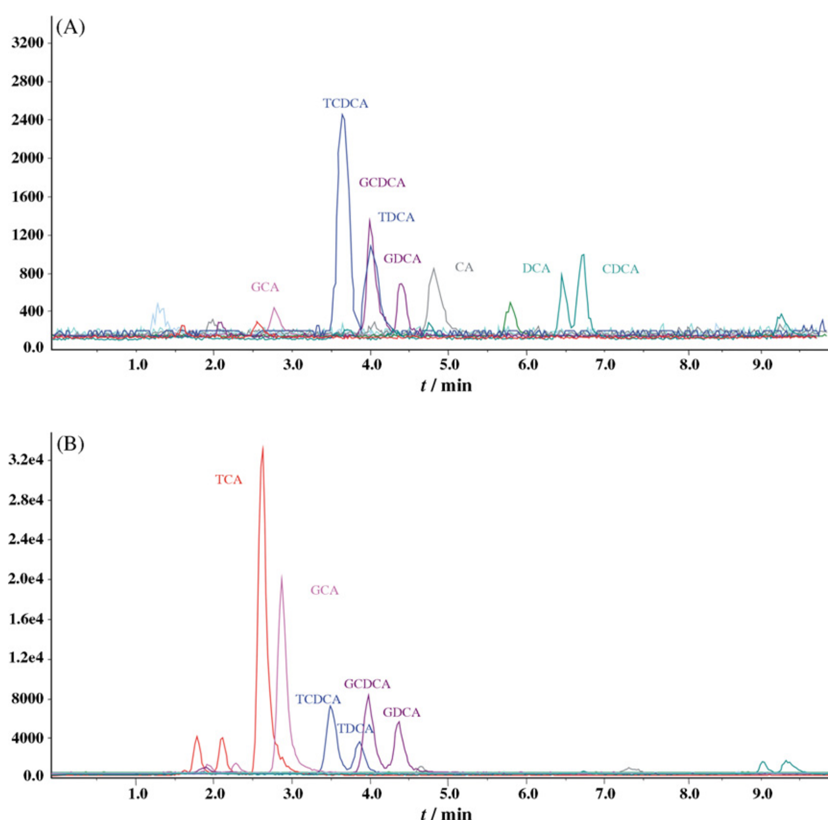

Fig. 4. Chromatograms of serum samples of a woman with healthy pregnancy (A) and a woman with ICP (B).

result [10]. In our study, we have found that the simple and efficient sample preparation method contributed to good reproducibility obtained. As discussed above, our sample preparation method did not involve protein precipitation and dissolve of dried residue, therefore minimized the potential sample loss and variations associated with protein precipitation and dissolve of the dried residue. In our SPE purification, compared to the method by Burkard et al. [16], we added a critical wash step with 45% methanol. This wash step was added to further removed less polar endogenous compounds which might interfere in the ionization efficiency of bile acids. We found that this wash step improved the mean recoveries and reproducibility of the bile acids analysis (65.3–121.5%, CVs ≤ 11.1% without the second wash step compared to 86.0–110%, CVs 6.9% with the second wash step). Furthermore, overload of the 5 μL injection loop with 20 μL of sample ensured that 5 μL of each sample was injected with high reproducibility. As a result of the application of our simple and efficient sample preparation method, we achieved satisfactory reproducibility in the analysis of 15 bile acids using external standards. As shown in Table 2, the within-day CVs were ≤3.8% and the between-day CVs were ≤6.1% for all bile acids studied. This degree of reproducibility is not inferior to published method using internal standards [16], and it is very suitable for potential applications in clinic analysis.

3.4. Application

The method was applied to the bile acids profile analysis of serum of 53 women with healthy pregnancies and 43 women with ICP. Representative MRM chromatograms of serum of a healthy woman and a patient with ICP are shown in Fig. 4. Significantly higher signals for conjugated bile acids were detected from the sample of the patient with ICP. Summarized results from the analysis of serum of all controls and ICP patients were shown in Fig. 5. While the detected levels of free bile acids for healthy pregnancy are comparable with the values from the literature [17], the concentrations of TCA, GCA, TUDCA, GCDCA and total bile acids in serum of women with ICP were significantly higher than those of healthy pregnancy controls. The elevated conjugated bile acids have been reported in serum from patients with liver disease [16,17]. As ICP patients usually have abnormal liver functions, the increased levels of conjugated bile acids in ICP patients in our study are not a surprise. Whether the increased conjugated bile acid in serum have any diagnostic value for ICP is still hard to evaluate at the monument. A carefully designed study involving a large number of samples has to be performed to answer above question. The finding is similar to previously reported result from Burkard et al. [16] by LC–MS/MS using an internal standard curve method and Sjovall's group obtained by GC–MS [17]. However, the cost of our method is much less, and it is simple and rapid. Although we only analyzed a limited number of samples, our results indicated that a profile analysis of bile acids could be a very effective tool for detection of metabolism alterations. It is also possible to extend this method to analysis of other bile acid like bile acid sulphates with minima modification of the method and work towards this is included in our ongoing study.

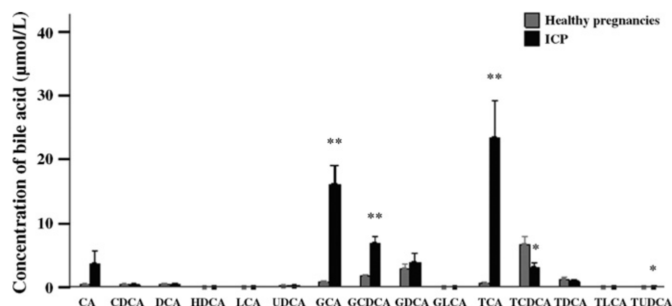

Fig. 5. Comparison of bile acid concentration in serum from healthy pregnancies and ICP. Values present the mean ± S.E.M. for the two groups. * indicates $P < 0.05$; ** indicates $P < 0.01$ compared with healthy pregnancies.

4. Conclusion

In this study we have developed a reliable LC–MS/MS method for simultaneous determination of free and conjugated bile acids in serum using external standard calibration method. The unique sample preparation method improves the reproducibility so that an external standard curve method could be used with satisfactory precisions. We demonstrate that the external standard method is suitable for quantitation of 15 bile acids in serum samples. The method is accurate, sensitive, rapid, specific and suitable for routine clinical practice and research practice.

Acknowledgement

This study has been supported by the National Natural Science Foundation of China (200475069).

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