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A simple and reliable bile acid assay in human serum by LC-MS/MS

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Abstract

Background: Bile acids, as important signaling molecules and regulatory factors acting on glucose, lipid, and energy metabolism, are always involved in liver, biliary, and intestinal diseases. Development and validation of a simple liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for determination of bile acids is significant for the routine clinical testing.

Methods: Fifty microlitre of serum was mixed with 10 µl of the internal standard working solution and then 140 µl of methanol for protein precipitation. After centrifuged, the supernatant was directly used for LC-MS/MS analysis.

Results: Good separation of all bile acid species was achieved. The method was validated with consistent linearity for individual bile acids, good recovery, low carryover, satisfactory sample stability, and analytical specificity against hemolysis, lipemia, and bilirubinemia. The intra-day and the inter-day imprecision values were in the range of 1.53%–10.63% and 3.01%–13.98%, respectively. No obvious matrix effect was observed. The reference intervals of bile acids in adults have been established for the clinical testing.

Conclusions: The low sample volume, simple sample preparation, good separation of all species, and satisfying validation results make this LC-MS/MS approach suitable for usage as a high-throughput assay in routine clinical laboratories.

KEYWORDS
bile acids, clinical testing, LC-MS/MS, serum

1 | INTRODUCTION

Bile acids play pivotal roles in the elimination of cholesterol, absorption of fat, and regulation of energy expenditure, and glucose and lipid metabolism.[1-4] In human, most abundant bile acids consist of primary bile acids (cholic acid, CA, and chenodeoxycholic acid, CDCA) and secondary bile acids (deoxycholic acid, DCA; lithocholic acid, LCA; ursodeoxycholic acid, UDCA).[3] The primary bile acids are synthesized from cholesterol exclusively in the liver via either the classic or alternative pathways[5] and account for the generation of about 90% of total bile acids in physiological settings.[6] The secondary bile acids were generated from primary bile acids by gut bacteria in the intestine.[7] The biosynthesis of bile acids usually terminates with the conjugation of glycine or taurine to form conjugated bile acids, such as glycocholic acid (GCA) and taurocholic acid (TCA).[8] Most bile acids are found within the liver and intestine and can be actively reabsorbed throughout the intestinal tract and delivered back to the liver in a process known as enterohepatic circulation.[7] As a result,

bile acid synthesis is tightly controlled by feedback mechanisms in health, and bile acids are implicated in many disease states, such as cholestasis, hepatic and intestinal cancers, liver cirrhosis, and diabetes mellitus.[9] Their multiple physiological, pathological, and pharmacological functions have kept bile acids as a research focus all the time. There are many differences in bile acid composition in various fluids and tissues due to the recycling of bile acids in the body. In human blood, CA, CDCA, DCA, LCA, and UDCA, as primary and secondary bile acids, are dominated. Among these, CA, CDCA, and DCA are mainly unconjugated.[6] The conjugated ones primarily exist in the amidated (mostly glycine and taurine) forms,[10] among which GCA is well-known for diagnosis of intrahepatic cholestasis of pregnancy,[11] and TCA is correlated well with liver injury.[12] As a result, simultaneous determination of CA, CDCA, DCA, LCA, UDCA, GCA, and TCA levels in human body is significant for monitoring of many diseases.

Up to now, many analytical methods have been developed to quantify BA concentrations in different kinds of biological samples. Enzymatic methods, due to their great simplicity and availability, have been predominant in the determination of bile acids up to the present day but may underestimate the total bile acid concentration.[13,14] Considering that the study of BA functions requires methods which cover the structurally diverse group of molecules, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has drawn great attention to quantify bile acids owing to its high sensitivity and specificity for distinguishing structural analogues.[15–24] However, most of these studies necessitated a dryness process in sample preparation by protein precipitation or solid phase extraction (SPE). This dryness process is time-consuming not favorable for rapid analysis in routine laboratories and is a source of potential errors due to the uncertainty in the dryness and re-dissolution process. As a result, sample preparation for determination of bile acids as simple as possible is significant in clinical testing for the characterization of the role that bile acids play in pathology.

The aim of this study was to validate a simple and reliable LC-MS/MS method in our laboratory for determination of serum bile acids. The primary reference intervals in a reference population of 130 volunteers have been further established.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Glycocholic acid hydrate (97%, Cat# G2878-500MG), lithocholic acid (≥95%, Cat# L6250-25G), ursodeoxycholic acid (≥99%, Cat# U5127-1G), taurocholic acid sodium salt hydrate (≥95%, Cat#T4009-1G), chenodeoxycholic acid (≥96%, Cat# C9377-100MG), lithocholic acid-D4 (LCA-D4, 98%, Cat# 589349-500MG), vanillylmandelic acid (VMA, ≥98%, Cat# H0131-500MG), and homovanillic acid (HVA, fluorimetric reagent, Cat# H1252-1G) were purchased from Sigma-Aldrich. Cholic acid-D4 (CA-D4, 98%, Cat# DLM-2611-0.05), deoxycholic acid-D4 (DCA-D4, 98%, Cat# DLM-2824-C), taurocholic acid sodium salt-D4 (TCA-D4, 98%,

Cat# DLM-9572-0.01), glycocholic acid-D4 (GCA-D4, 98%, Cat# DLM-2742-C), and chenodeoxycholic acid-D5 (CDCA-D5, 98%, Cat# DLM-9327-0.05) were purchased from Cambridge Isotopes. Ursodeoxycholic acid-D4 (UDCA-D4, Cat# U-002-1 ml) was bought from Supelco, Inc. Cholic acid (Cat# C432600), deoxycholic acid (Cat# D232645), eicosapentaenoic acid (EPA, Cat# E477800), and docosahexaenoic acid (DHA, Cat# D494500) were obtained from TRC. Formic acid (99%, LC-MS grade, Cat# A117-50) was obtained from Fisher Scientific. Methanol (HPLC grade, ≥99.9%, Cat# 34885, expire date 2024/03/31) and acetonitrile (HPLC grade, ≥99.9%, Cat# 34851, expire date 2023/03/31) were bought from Merck. Interference Check A (REF ZG900133, Lot ZR0501, expire date 2022/08/04) was supplied by Sysmex Co., LTD. When purity and expire date were not found on the reagent boxes or in the reagent instructions, they were not indicated here. Deionized water was freshly prepared by a water purification system (ELGA).

2.2 | LC-MS/MS conditions

The LC-MS/MS analysis was performed on a AB Sciex Exion LC coupled to a Qtrap 5500 (Applied Biosystems/Sciex). The LC system was consisted of a gradient pump, a vacuum degasser, a temperature-controlled autosampler, and a temperature-controlled column oven. A Thermo Hypersil Gold C18 (2.1 × 100 mm, 1.9 μm) column was used for separation. The mobile phase consisted of solvent A (water containing 0.1% FA) and solvent B (methanol and acetonitrile at a ratio of 2:1 v/v containing 0.1% FA). Gradient elution was achieved as follows: 0–6.0 min, 50%B–72%B; 6.0–14.00 min, 72%B–80%B; 14.01–15.50 min, keeping 100%B and finally running 50%B for re-equilibration of the column with 1.5 min. The flow rate was 0.3 ml/min. The column temperature was 40°C, and the sample injection volume was 10 μl. Between injections, the autosampler syringe was washed with methanol.

The Qtrap 5500 was operated in negative mode. The optimal MS conditions were as follows: ion spray voltage of −4500 V, temperature of 450°C, curtain gas of 30.0 psi, ion source gas 1 of 40 psi, and ion source gas 2 of 50 psi. The specific selected ion monitoring (SIM) or multiple reaction monitoring (MRM) transitions for quantification under optimal conditions were monitored as shown in Table S1.[23] The MS system was controlled by a Analyst 1.6.1 software.

2.3 | Preparation of solutions, calibrators, and quality controls (QCs)

Stock solutions of glycocholic acid (1.85 mM), lithocholic acid (2.90 mM), ursodeoxycholic acid (2.29 mM), taurocholic acid sodium salt hydrate (2.11 mM), chenodeoxycholic acid (2.40 mM), cholic acid (4.85 mM), and deoxycholic acid (4.69 mM) were prepared in methanol and equally mixed to generate a analyte working solution. The stable isotope internal standards (IS) LCA-D4 (29.96 μM), UDCA-D4 (12.60 μM), GCA-D4 (10.65 μM), TCA-D4 (18.46 μM), CDCA-D5

(22.64 μM), CA-D4 (31.70 μM), and DCA-D4 (12.60 μM) dissolved in methanol were equally mixed to generate a IS working solution. All above working solutions were stored at −40°C until analysis. It is noted that all set-ups used for standard solution preparation including glassware, pipettes, and analytical balance are calibrated annually according to the College of American Pathologists checklist for our lab.

A 9-point calibration curve (3.238–1034 nM for LCA, 25.53–8170 nM for UDCA, 20.61–6595 nM for GCA, 23.58–7545 nM for TCA, 26.85–8590 nM for CDCA, 54.14–17325 nM for CA, and 52.35–16750 nM for DCA) was constructed by serial dilution of the mixed analyte working solution with the blank serum. The calibrators were freshly prepared before each experiment. Considering that the commercial charcoal stripping serum bought from SeraCare Life Sciences (#22011) was only removal of fibrinogen, lipids, and hormone, this commercial charcoal stripping serum still has measurable bile acids. This bile acid-depleted serum was prepared by the Oasis HLB cartridges (3 ml/60 mg, Waters) extracting the serum purchased form SeraCare Life Sciences, when bile acids were absorbed onto the sorbent. An aliquot of the eluent was collected and then analyzed to ensure that no bile acids could be detected.

Quality controls were prepared in the blank serum within the expected biological range at two levels (32.32 and 517.2 nM for LCA, 255.3 and 4085 nM for UDCA, 206.1 and 3298 nM for GCA, 268.4 and 4295 nM for CDCA, 230.8 and 3772 nM for TCA, 541.4 and 8662 nM for CA, and 523.5 and 8375 nM for DCA). The biological range levels were selected according to the reference intervals reported by Mayo Clinic Laboratories.[25] The level of QCL for each analyte was below the reference interval and QCL was denoted as the normal range control, while the level of QCH was above the reference interval and QCH was denoted as the pathological range control.

In addition, a pooled serum specimen prepared by mixing real human serum samples, as a pooled quality control (PQC), was used as an additional quality control for good daily monitoring. The PQC was aliquoted and stored at −80°C until use. The target concentrations of PQC (6.519 nM for LCA, 53.91 nM for UDCA, 412.6 nM for GCA, 809.8 nM for CDCA, 70.92 nM for TCA, 1421 nM for CA, and 185.7 nM for DCA) were assigned by successive determination of PQC at least 15 times. QCs and PQC were analyzed at the beginning, middle, and end of each analytical run.

2.4 | Sample collection and preparation

To investigate the influence of serum collection tubes on testing of bile acids, five subjects were selected for blood collection in both serum separator tubes with and without separator gel, and the concentrations of bile acids were compared. For clinical testing, blood samples were collected into yellow tubes with separator gel. After 1 h at room temperature for clotting, the samples were centrifuged at $850 \times g$ for 5 min.

Calibrators/QCs/serum specimens (50 μl) were mixed with 10 μl of the IS working solution, and then, 140 μl of methanol was added into tubes for protein precipitation. The tubes were vortexed for 3 min and then centrifuged for 10 min at $20238 \times g$. The supernatants were used for direct analysis.

2.5 | Method validation

The LC/MS/MS method was validated emphasizing on linearity, the lower limit of quantification (LLOQ), the limit of detection (LOD), precision, accuracy, sample stability, carryover, and analytical specificity according to the European Medicines Agency guideline on bioanalytical method validation (European Medicines Agency 2011).

Linearity was evaluated by analysis of a 9-point calibration curve. The calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards must fulfill this criterion. The LLOQ, defined as the lowest concentration with a signal-to-noise ratio >10, was determined by assaying a series of low-concentration calibrators in five replicates and established based on the criteria that a coefficient of variation (CV) of <20% and an accuracy within 80%–120%. The LOD represents the concentration that gives a signal-to-noise ratio >3.

Quality controls and PQC were used to evaluate imprecision. Five replicates of each level were assayed over five days to evaluate the intra-day and inter-day precision. The CVs were within the accepted validation criteria of less than 20% at the LLOQ and less than 15% at all other tested concentrations.

Accuracy of the method was evaluated by a recovery study. In this experiment, human serum spiked with known amounts of bile acids at four levels (13.0, 51.8, 207, and 414 nM for LCA, 102, 408, 1635, and 3270 nM for UDCA, 82.5, 330, 1320, and 2640 nM for GCA, 107, 430, 1720, and 3440 nM for CDCA, 94.5, 378, 1510, and 3020 nM for TCA, 216, 866, 3465, and 6930 nM for CA, and 209, 838, 3350, and 6700 nM for DCA) were assayed in five replicates at each level. The recovery was calculated as [(final concentration-initial concentration)/added concentration].

Matrix effect (ME) was evaluated by two methods. First, a post-column infusion study was performed by injecting the blank serum matrix without IS onto the LC column while the analyte solution containing IS at a flow rate of 10 μl/min was injected into MS. Secondly, a signal-recovery spiking experiment was performed by comparing signals of standard substances added to the blank serum matrix (25.9, 51.8, 207, and 414 nM for LCA, 204, 408, 1634, and 3268 nM for UDCA, 165, 330, 1319, and 2638 nM for GCA, 189, 377, 1509, and 3019 nM for TCA, 215, 430, 1718, and 3436 nM for CDCA, 433, 866, 3465, and 6930 nM for CA, and 419, 838, 3350, and 6700 nM for DCA) with the signal of that added to methanol at the same level. The absolute matrix factor (MF) of the analyte and IS is calculated as Ai/Ai' × 100% (Ai and Ai' represent peak area of the analyte or IS within and without matrix, respectively), and the IS-normalized matrix factor (IS-normalized MF) of the analyte is calculated as (MF

of analyte) / (MF of IS) × 100%.[26] An IS-normalized MF within 85%-115% could be accepted.

Carryover was evaluated by spiking the blank serum with bile acids (827.6 nM LCA, 6536 nM UDCA, 5276 nM GCA, 6036 nM TCA, 6872 nM CDCA, 13860 nM CA, and 13400 nM DCA) at a ratio of 95:5 (v/v) as the high concentration sample, and methanol was assigned as the blank sample. The high concentration sample and the blank sample were injected in alternative for five times. Carryover is not significant if the signal of any peak in the blank sample at the retention time of bile acids is below the signal of LOD.

The pooled serum specimen was aliquoted and stored at room temperature, in a refrigerator (4°C) or in a freezer (−80°C). The bile acids level was determined at different time intervals (0, 1, 2, 3, 7, 14, 21, and 28 days). Five replicates were measured at each time point under different conditions. The QCs were subjected to three freeze–thaw cycles, and the freeze–thaw stability of QCs at three levels was evaluated.

The assay interference was investigated by an interference check A kit which consisted of bilirubin•F (3078–3762 μmol/L), bilirubin•F (blank), bilirubin•C (3078–3762 μmol/L), bilirubin•C (blank), hemolytic hemoglobin (45–55 g/L), hemolytic hemoglobin (blank), Chyle (14000–28000 FTU (Formazine turbidity unit)), and Chyle (blank). A pooled serum sample was mixed with each of the above kit components at a ratio of 9:1 (v/v). These samples were subjected to sample preparation for assaying interference in order to determine whether these interferents may have a positive or negative impact on measuring the true analyte concentration. In addition, additional endogenous acid compounds, such as VMA, HVA, EPA, and DHA, were tested for interference with the assay. The potential interferences (1.50 mM VMA, 1.62 mM HVA, 1.30 mM EPA, and 1.37 mM DHA) were spiked into QCs and PQC at a ratio of 1:9. Then, these samples together with an equivalent series of non-spiked samples were subjected to sample preparation for assaying interference.

2.6 | Serum levels of bile acids in the reference population

Serum specimens were obtained from healthy subjects ($n = 130$, 65 females and 65 males) with normal renal and hepatic function, normal lipid levels, and no underlying diseases. Study subjects had fasted overnight. The concentrations of bile acids for these subjects were measured using the new method on their collecting day.

2.7 | Statistics

Statistical analyses were carried out using SPSS Statistics 20.0 software package. Values below LOD were defined as LOD.[27] The test results from different types of blood collection tubes were compared by paired t test, and statistical significance was defined as $p < 0.05$.[28] The normality of the data was tested by a one-sample Kolmogorov–Smirnov test. The outermost 5% of observations were used to define limits for one-side reference intervals (95th for bile acids).

3 | RESULTS

3.1 | LC-MS/MS conditions

In recent years, LC-MS/MS has become the most suitable method for the quantification of bile acids due to its selectivity and specificity. Most bile acids represent structural analogues, differing only in position and stereo-specific configuration of hydroxyl groups at the steroid backbone as shown in Figure 1. Due to the lack of specific fragment ions, quantification of some bile acids was accomplished by monitoring the same m/z values for both the parent ion and the fragment ion, as reported in the literature.[23] In addition, due to the similar structures, good separation is a prerequisite for quantification of individual bile acids. After optimization of chromatographic conditions, LCA, UDCA, GCA, TCA, CDCA, CA, and DCA could be well separated on a Thermo Hypersil Gold C18 as shown in Figure 2. In company of the SIM or MRM parameters displayed in Table S1, the target bile acids in the present study could be quantified reproducibly and accurately.

3.2 | Sample collection and preparation

For clinical testing of bile acids by LC-MS/MS, types of blood collection tubes were investigated. Serum separator tubes with or without separator gel had no obvious influence on concentrations of bile acids (p all above 0.05, paired t test). In addition, the percentage differences of the testing results between the two collection tubes were in the range from −12.69% to 21.25% as displayed in Table S2. All these indicated that both types of blood collection tubes might be used in clinical tests. However, the type of blood collection tubes should be consistent in the routine clinical testing.

3.3 | Method validation

Linearity was determined by analysis of a 9-point calibration curve. Charcoal stripped serum after removal of bile acids was used as the blank serum matrix for preparation of calibrators. The linear correlation coefficients (r^2) of the calibration curves for all analytes above 0.9945 exhibited excellent linearity for quantification. A high linearity response over a sufficient dynamic concentration range was observed as summarized in Table 1. The inter-assay variability of the calibration data was ≤15.88% as shown in Table S3.

Analysis of a series of low-concentration calibrators resulted in an accuracy within 80%–120% and a CV value <20% at the concentration of 3.238, 12.75, 5.156, 26.85, 11.78, 54.14, and 26.12 nM for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA, respectively. As a result, 3.238, 12.75, 5.156, 26.85, 11.78, 54.14, and 26.12 nM were
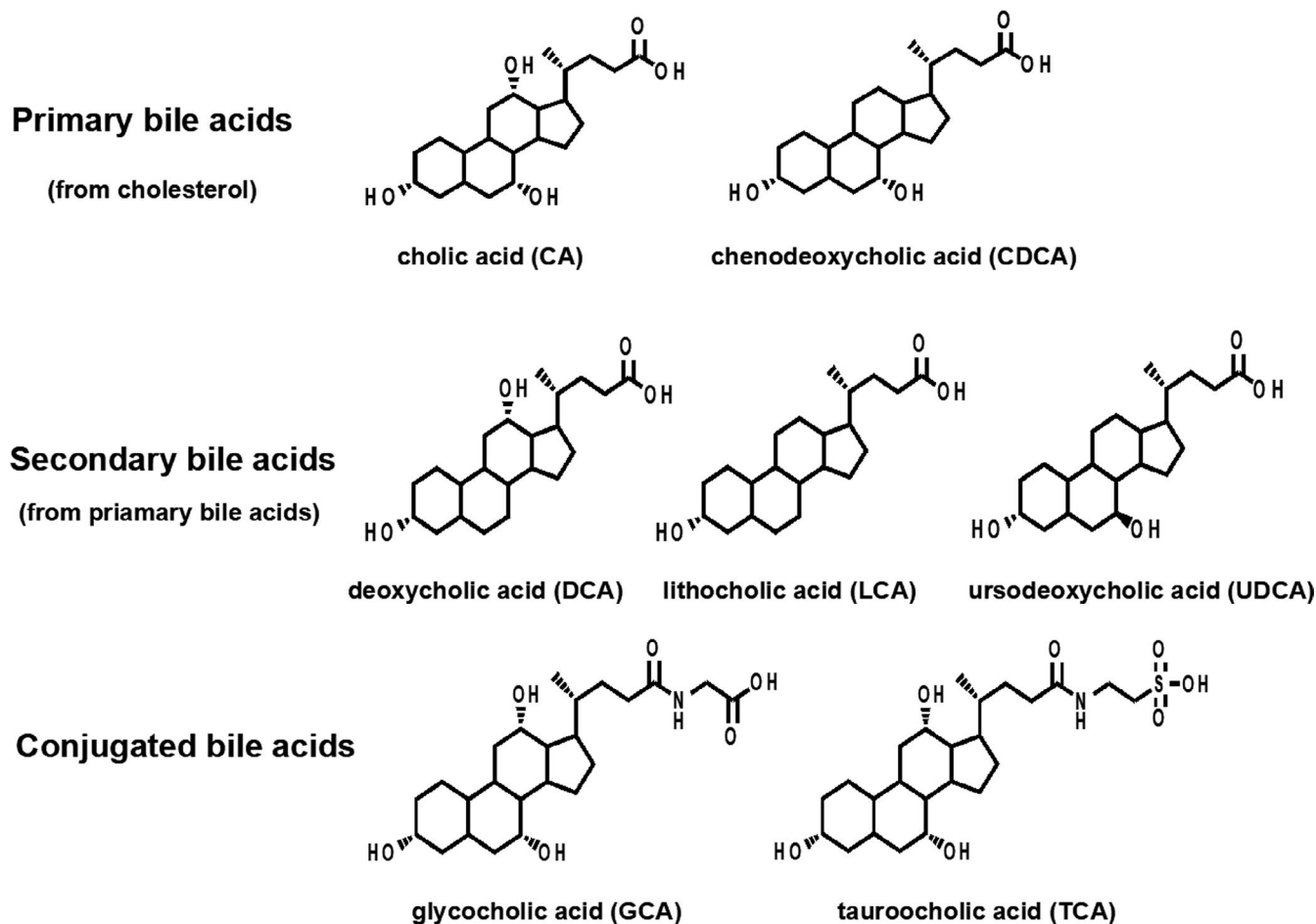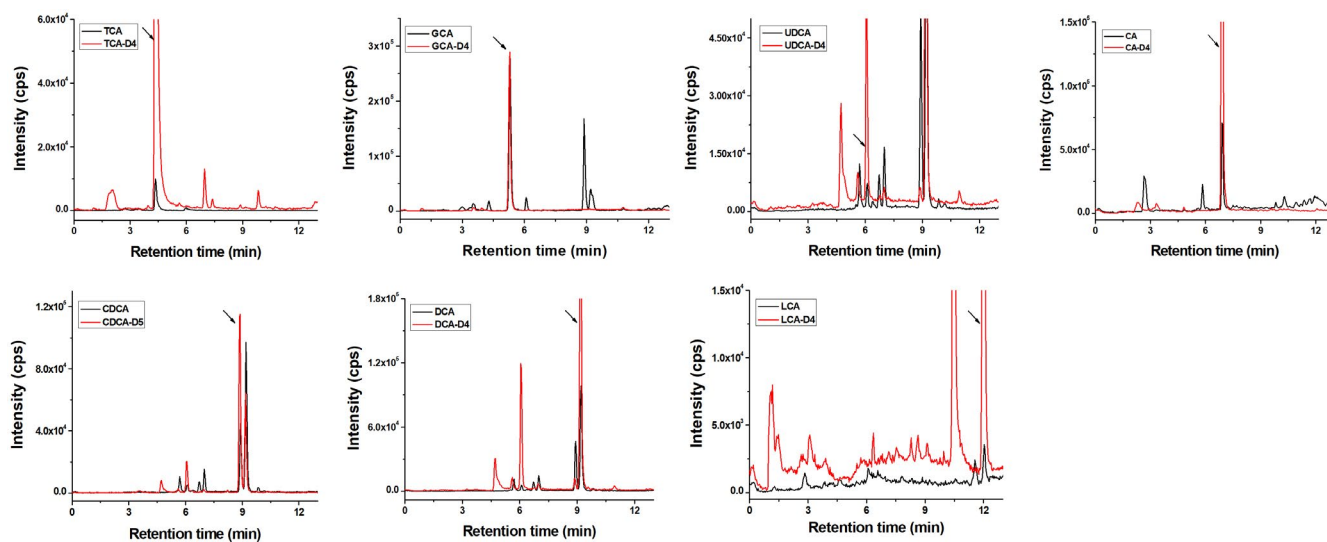
FIGURE 1 Structures of bile acids

FIGURE 2 Chromatogram of bile acids in human serum (TCA: 29.9 nM; GCA: 272 nM; UDCA: 30.2 nM; CA: 157 nM; CDCA: 347 nM; DCA: 200 nM; LCA: 7.84 nM)

accepted as the LLOQ with the signal-to-noise ratio >10 for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA, respectively. The concentration of 1.612, 6.375, 2.578, 13.38, 5.894, 27.07, and 13.06 nM for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA was considered as

the LOD with the signal-to-noise ratio >3. All results are presented in Table 1.

Imprecision was calculated as the CV for QCs prepared in the blank serum and PQC prepared from a serum pool. Intra-day CVs

TABLE 1 Linearity, LLOQ, and LOD

Analyte	Linear range (nM)	Linear equation	r^2	LLOQ (nM)	LOD (nM)
LCA	3.238–1034	y=0.00161x + 0.00434	0.9957	3.238	1.612
UDCA	25.53–8170	y=0.00354x + 0.0676	0.9954	12.75	6.375
GCA	20.61–6595	y=0.00443x + 0.015	0.9946	5.156	2.578
CDCA	26.85–8590	y=0.00149x + 0.059	0.9945	26.85	13.38
TCA	23.58–7545	y=0.00111x + 0.00604	0.9958	11.78	5.894
CA	54.14–17325	y=0.000884x + 0.0101	0.9948	54.14	27.07
DCA	52.35–16750	y=0.00181x + 0.0209	0.9946	26.12	13.06

were less than 10.63%, 6.58%, 2.84%, 8.86%, 3.04%, 2.59%, and 2.03%, while inter-day CVs were less than 13.98%, 10.02%, 7.66%, 11.21%, 7.29%, 8.34%, and 7.58% for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA, respectively (Table 2).

The serum pool was spiked of bile acids at four concentrations spanning the expected biological range. Individual data points of recovered concentration (final concentration-initial concentration) for at least five replicates at each concentration were compared to the spiked concentration. The CV values of recoveries spiked at four concentrations were in the range of 2.86%–8.46%, 3.81%–11.58%, 3.43–18.32%, 3.71%–14.37%, 3.65%–8.28%, 1.59%–11.28%, and 1.77%–5.90% for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA, respectively. Recovery data for LCA (83%–104%), UDCA (88%–98%), GCA (91%–115%), CDCA (89%–110%), TCA (96%–117%), CA (82%–114%), and DCA (87%–110%) are listed in Table 3.

The extent of ion suppression or ion enhancement was assessed by a post-column infusion study and a signal-recovery spiking experiment. For the former, no ion suppression or enhancement was found at the retention time of LCA, UDCA, GCA, CDCA, TCA, CA, and DCA (Figure S1). For the latter, the IS-normalized matrix factors at four different levels were in the range of 0.86–1.06, 0.93–1.10, 0.85–1.03, 1.01–1.24, 0.86–0.96, 0.93–1.07, and 1.01–1.25 with CVs ≤9.67% for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA, respectively (Table 4). All these results demonstrated that there was no significant ion suppression or ion enhancement at different analyte concentrations.

In evaluation of carryover, the high concentration sample and the blank sample were alternated for six times. At the retention time of individual bile acids, the average peak area associated with the blank sample was below average peak area of the LOD as shown in Table S4, indicating that no significant carryover was found.

Stability of human serum was assessed freshly and after storage under a certain condition at different time points (Day 1, 2, 3, 7, 14, 21, and 28). As displayed in Figure 3, the percentage differences, calculated as [(analyte concentrations after storage - analyte concentrations at Day 0) / analyte concentrations at Day 0] × 100%, were all within ±20%, indicating that the serum sample could be stable for at least one month at room temperature (RT), or in a refrigerator (4°C) or freezer (−80°C). The freeze–thaw stability of QCs at three levels were further evaluated. The percentage differences of LCA, UDCA, GCA, CDCA, TCA, CA, and DCA were in the range of −8.97% to 10.92%, −18.21% to 3.53%, −13.87% to 10.87%, −15.91% to −10.8%, −16.71% to 4.87%, −17.63% to 6.69%, and −14.83% to 2.98%, respectively. It is indicated that the concentrations of these bile acids could be determined as stable within three freeze–thaw cycles.

Analytical specificity of this method toward bilirubin, hemolytic hemoglobin, and chyle was evaluated. Percentage difference, calculated as [(analyte concentrations in the sample spiked with the interferent - analyte concentrations in the blank sample) / analyte

TABLE 2 Imprecision

Analyte	Concentration (nM)	Intra-day (%CV)	Inter-day (%CV)
LCA	QCL	2.29	6.59
	QCH	3.62	9.50
	PQC	10.63	13.98
UDCA	QCL	2.82	6.70
	QCH	4.16	7.93
	PQC	6.58	10.02
GCA	QCL	2.79	4.29
	QCH	2.84	7.66
	PQC	1.53	4.37
CDCA	QCL	8.86	11.21
	QCH	2.30	6.59
	PQC	3.36	8.44
TCA	QCL	1.67	4.61
	QCH	3.04	7.29
	PQC	1.71	3.61
CA	QCL	2.23	4.49
	QCH	2.09	8.34
	PQC	2.59	4.00
DCA	QCL	2.03	3.76
	QCH	1.76	7.58
	PQC	2.02	3.01

Note: Although the intra-day or inter-day CVs for LCA, UDCA nd CDCA were above 10%, they were all below 15% and could meet the requirement of quantification.

TABLE 3 Recovery

Analyte	Spiked concentration (nM)	Recovery (%)	Recovery-baseline* (%)	CV (%)
LCA	13.0	102	2	8.46
	51.8	83	−17	2.86
	207	92	−8	3.10
	414	104	4	3.74
UDCA	102	96	−4	11.58
	408	88	−12	3.81
	1635	89	−11	4.13
	3270	98	−2	5.82
GCA	82.5	98	−2	18.32
	330	91	−9	8.90
	1320	102	2	3.43
	2640	115	15	6.96
CDCA	107	110	10	14.37
	430	91	−9	3.71
	1720	89	−11	5.22
	3440	102	2	5.08
TCA	94.5	97	−3	8.28
	378	96	−4	4.16
	1510	111	11	3.65
	3020	117	17	4.21
CA	216	114	14	11.28
	866	109	9	1.59
	3465	85	−15	3.88
	6930	82	−18	4.64
DCA	209	110	10	5.90
	838	93	−7	1.77
	3350	87	−13	2.62
	6700	96	−4	5.04

Note: *100% is used as the recovery baseline.

The recovery was evaluated at four levels. At the lowest level, the recoveries of UDCA, GCA, CDCA and CA were between 11.28% and 18.32%.

TABLE 4 Matrix effect

Analyte	Concentration (nM)	IS-normalized matrix factor	CV (%)
LCA	25.9	0.95	1.86
	51.8	1.06	4.14
	207	0.86	3.91
	414	1.06	4.34
UDCA	204	0.93	6.51
	408	1.04	4.61
	1634	1.03	9.67
	3268	1.10	8.13
GCA	165	0.85	3.47
	330	0.90	5.09
	1319	0.93	4.16
	2638	1.03	4.57
CDCA	215	1.24	4.92
	430	1.01	4.40
	1718	1.12	1.95
	3436	1.19	3.39
TCA	189	0.86	2.71
	377	0.91	4.88
	1509	0.89	5.18
	3019	0.96	6.30
CA	433	0.93	4.42
	866	1.00	3.92
	3465	1.01	6.27
	6930	1.07	7.18
DCA	419	1.25	4.60
	838	1.01	3.72
	3350	1.11	2.75
	6700	1.18	2.65

concentrations in the blank sample] × 100%, was employed to evaluate interference. As demonstrated in Table 5, bilirubin, hemoglobin, and lipids had no obvious effect on results with percentage differences within ±20% except that hemoglobin has slightly effect on CA determination and lipids pose a significant influence on TCA measurement. As a result, hemolysis and lipemia is warranted for determination of CA and TCA, respectively. In addition, after the addition of other acid compounds to the low control, high control, and PQC, average biases of bile acids were in the range of 8.23%–13.40% for LCA, 11.29%–14.56% for UDCA, 7.71%–17.24% for GCA, 1.07%–3.12% for CDCA, 1.17%–1.55% for TCA, 1.35%–2.60% for CA, and 9.60%–13.19% for DCA, respectively. Furthermore, no interference peak was observed at the retention time of bile acids.

3.4 | Serum levels of bile acids in the reference population

Bile acids are formed in the liver from cholesterol, conjugated primarily to glycine and taurine, stored and concentrated in the gallbladder, and secreted into the intestine after the ingestion, and then approximately 90% of the bile acids are reabsorbed throughout the enterohepatic circulation. While elevated serum levels of bile acids are the hallmark of cholestasis, bile acid concentrations are also elevated in patients with acute hepatitis and with liver cirrhosis.[29] Considering that the elevation of bile acids had great clinical significance, the upper limits of reference intervals or cutoff values were established by non-parametric 95th percentile based on the abnormally distributed data. Serum specimens were collected from healthy subjects (n = 130, 65 F, 65 M, mean age 41 y, range 20–67 years). The upper limit of RIs for LCA, UDCA, GCA, CDCA, TCA, CA, and DCA was 33.7, 708, 968, 2414, 85.8, 1422, and 707 nM, respectively. In future, a large sample size should be included for RIs investigation.
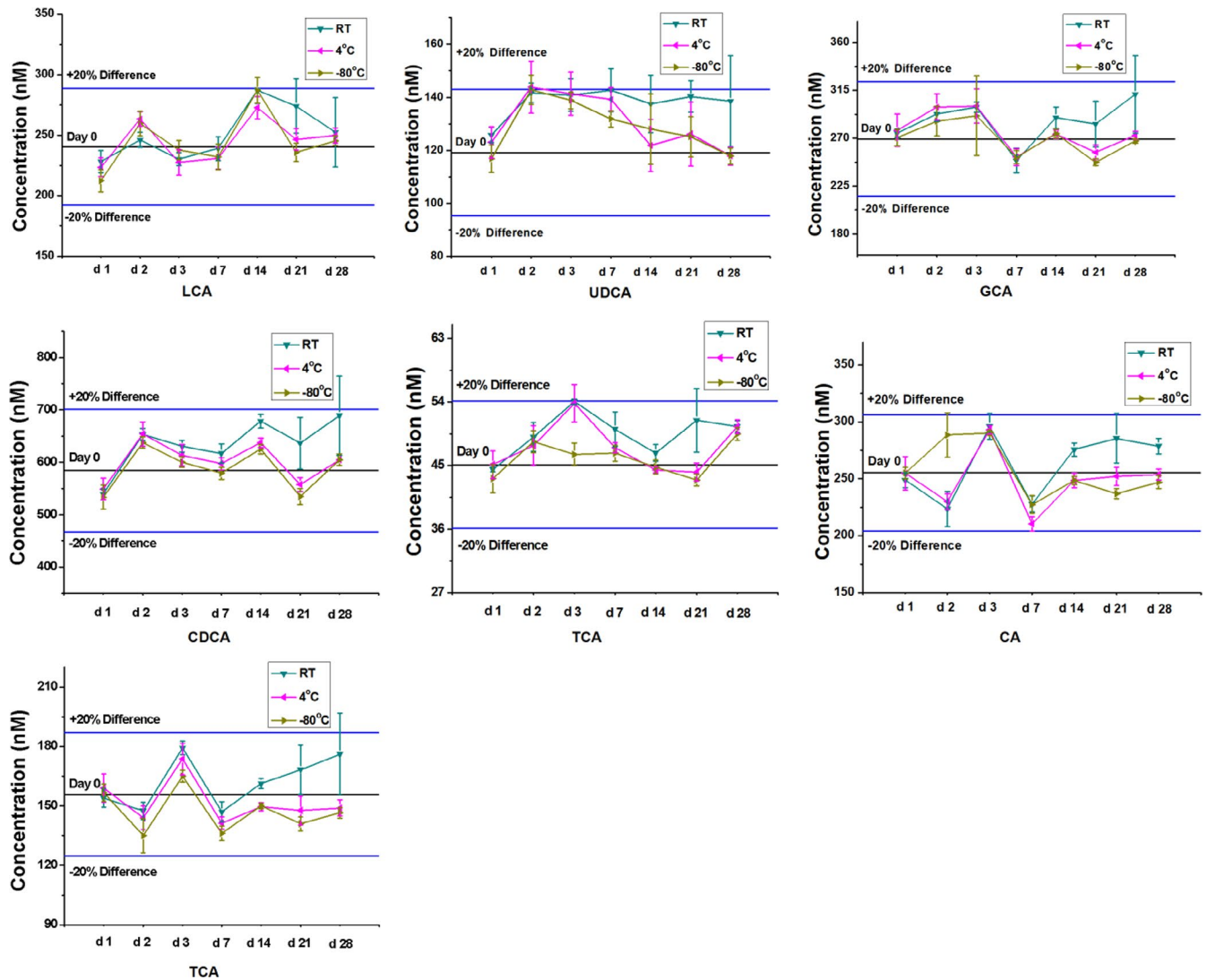
FIGURE 3 Stability of serum samples

TABLE 5 Analytical specificity

	Percentage difference (%)			
Analyte	Bilirubin, free	Bilirubin, combine	Hemolytic hemoglobin	Chyle
LCA	−6.80	9.48	−2.02	4.67
UDCA	−2.98	6.02	−6.64	−3.66
GCA	−4.33	7.78	−2.37	3.28
CDCA	−1.01	10.70	−2.64	3.36
TCA	−3.41	10.42	−4.12	78.98
CA	−0.40	16.83	31.8	7.98
DCA	−2.84	15.42	−2.71	1.98

Note: The percentage differences of CDCA, TCA, CA and DCA were in the range of 10.42-16.83%, but within ±20%. As a result, bilirubin has no obvious impact on detetmination of all analytes except that hemoglobin has slightly affects on CA determination (31.8%) and lipids pose a significant influence on TCA (78.98%).

4 | DISCUSSION

Disruption of normal bile acids synthesis and metabolism is associated with cholestasis, gallstones, inflammation, and bacterial overgrowth, and so on. Bile acids are mainly composed of CA, CDCA, DCA, LCA, and UDCA and could be subsequently amidated with taurine or glycine.[30] As the most famous and mature biomarkers in intrahepatic cholestasis of pregnancy and liver injury,[11,12] GCA and TCA together with CA, CDCA, DCA, LCA, and UDCA were analyzed in this study. Herein, we report the development and validation of a LC-MS/MS methods for bile acids analysis in adults that requires a low sample volume and simple sample preparation. Good separation of bile acids in human serum was achieved as a result of application of 1.8 μm C18 column on basis of a mobile phase partially composed of organic solvent mixtures without salts. In consideration of analyte loss through sample preparation and matrix effect, quantification of bile acids was accomplished by the use of commercially available isotopically labeled standards. For sample preparation, SPE has

often been used for the extraction of bile acids from complex matrix. However, unsatisfying recovery rates and poor reproducibility values were observed in previously described SPE procedures.[20] As a result, protein precipitation (PP) method has become an alternative to SPE method. However, in most PP procedures, the supernatants should be collected for dryness and re-dissolution which is time-consuming. Herein, a simple one-step PP process based on good separation of bile acids and high sensitivity of the instrument has been developed for successful measurement of bile acids, even though the LCA has a lower physiological concentration.[25] In this pre-treatment process, dryness was not needed and the supernatant could be used for direct LC-MS/MS analysis. This simple sample pre-processing step could ensure its robust application in clinical laboratories.

A potential beneficial use of our method for the investigation of bile acid levels in human serum in clinical laboratories was further ensured by full validation of this LC-MS/MS methods with consistent linearity (r^2 between 0.9945 and 0.9958), satisfactory imprecision (within 1.53%–13.98%), and accuracy (between 82% and 117%). The established reference intervals of bile acids in adults were comparable to that in the Mayo Clinic Laboratories.[25]

Despite of the satisfactory analytical performance as discussed above, our study does suffer from the following limitations: firstly, the number of bile acid species is limited; secondly, no commercial quality controls were available and rigorous concordance study was not performed to verify inter-laboratory accuracy.

5 | CONCLUSION

In summary, a simple and reliable LC-MS/MS method for the simultaneous determination of bile acids in human serum has been developed and validated. Improvements in comparison with previous LC-MS/MS methods were achieved regarding simple sample preparation without a dryness process, good separation of bile acids as a result of application of 1.8 μm C18 column based on a mobile phase partially composed of organic solvent mixtures. The primary reference intervals in adults were established for clinical use. This LC-MS/MS method could be potentially applied in clinical laboratories.

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REFERENCES

1. Camilleri M, Gores GJ. Therapeutic targeting of bile acids. *Am J Physiol Gastrointest Liver Physiol*. 2015;309:G209-G215.
2. Perino A, Demagny H, Velazquez-Villegas L, Schoonjans K. Molecular physiology of bile acid signaling in health, disease, and aging. *Physiol Rev*. 2021;101:683-731.
3. Bathena SP, Thakare R, Gautam N, et al. Urinary bile acids as biomarkers for liver diseases I: stability of the baseline profile in healthy subjects. *Toxicol Sci*. 2015;143:296-307.
4. Boesjes M, Brufau G. Metabolic effects of bile acids in the gut in health and disease. *Curr Med Chem*. 2014;21:2822-2829.
5. Chiang JY. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev*. 2002;23:443-463.
6. Fiorucci S, Distrutti E, Carino A, Zampella A, Biagioli M. Bile acids and their receptors in metabolic disorders. *Prog Lipid Res*. 2021;82:101094.
7. Li TG, Chiang JY. Regulation of bile acid and cholesterol metabolism by PPARs. *PPAR Res*. 2009;2009:1-15.
8. Shiffka SJ, Kane MA, Swaan PW. Planar bile acids in health and disease. *Biochim Biophys Acta Biomembr*. 2017;1859:2269-2276.
9. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci*. 2008;65:2461-2483.
10. Bathena SPR, Mukherjee S, Olivera M, et al. The profile of bile acids and their sulfate metabolites. *J Chromatogr B*. 2013;942:53-62.
11. Fu C, Xu YL. Value of serum glycocholic acid and total bile acids in predicting maternal and perinatal outcomes in intrahepatic cholestasis of pregnancy. *J. Healthc Eng*. 2021;2021:3911998.
12. Luo L, Schomaker S, Houle C, Aubrecht J, Colangelo JL. Evaluation of serum bile acid profiles as biomarkers of liver injury in rodents. *Toxicol Sci*. 2014;1:12-25.
13. Žížalová K, Vecka M, Vítek L, Leníček M. Enzymatic methods may underestimate the total serum bile acid concentration. *PLoS One*. 2020;15:e0236372.
14. Danese E, Salvagno GL, Negrini D, Brocco G, Montagnana M, Lippi G. Analytical evaluation of three enzymatic assays for measuring total bile acids in plasma using a fully-automated clinical chemistry platform. *PLoS One*. 2017;12:e0179200.
15. Scherer M, Gnewuch C, Schmitz G, Liebisch G. Rapid quantification of bile acids and their conjugates in serum by liquid chromatography-tandem mass spectrometry. *J Chromatogr B*. 2009;877:3920-3925.
16. Steiner C, von Eckardstein A, Rentsch KM. Quantification of the 15 major human bile acids and their precursor 7α-hydroxy-4-cholesten-3-one in serum by liquid chromatography-tandem mass spectrometr. *J Chromatogr B*. 2010;878:2870-2880.
17. Batherna SPR, Mukherjee S, Olivera M, Alnouti Y. The profile of bile acids and their sulfate metabolites in human urine and serum. *J Chromatogr B*. 2013;942:53-62.
18. Han J, Liu Y, Wang RX, Yang JC, Ling V, Borchers CH. Metabolic profiling of bile acids in human and mouse blood by LC-MS/MS in combination with phospholipid-depletion solid-phase extraction. *Anal Chem*. 2015;87:1127-1136.
19. Si GL, Yao P, Shi L. Rapid determination of bile acids in bile from various mammals by reversed-phase ultra-fast liquid chromatography. *J Chromatogr Sci*. 2015;53:1060-1065.
20. Wegner K, Just S, Gau L, et al. Rapid analysis of bile acids in different biological matrices using LC-ESI-MS/MS for the investigation of bile acid transformation by mammalian gut bacteria. *Anal Bioanal Chem*. 2017;409:1231-1245.
21. Yang TT, Shu T, Liu GL, et al. Quantitative profiling of 19 bile acids in rat plasma, liver, bile and different intestinal section contents to investigate bile acid homeostasis and the application of temporal variation of endogenous bile acids. *J Steroid Biochem Mol Biol*. 2017;172:69-78.

22. Luo L, Aubrecht J, Li D, et al. Assessment of serum bile acid profiles as biomarkers of liver injury and liver disease in humans. *PLoS One.* 2018;13:e0193824.

23. Fu XW, Xiao Y, Golden J, Niu SZ, Gayer CP. Serum bile acids profiling by Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) and its application on pediatric liver and intestinal diseases. *Clin Chem Lab Med.* 2020;58:787-797.

24. Gómez C, Stücheli S, Kratschmar DV, Bouitbir J, Odermatt A. Development and validation of a highly sensitive LC-MS/MS method for the analysis of bile acids in serum, plasma, and liver tissue samples. *Metabolites.* 2020;10:282.

25. Mayo Clinc Laboratories. BAPS-bile acid profile, serum. 2021. https://www.mayocliniclabs.com/test-catalog/Overview/62538 Accessed 30 November, 2021

26. Shen Y, Luo X, Li HJ, Chen ZJ, Guan Q, Cheng LM. Simple and reliable serotonin assay in human serum by LC-MS/MS method coupled with one step protein precipitation for clinical testing in patients with carcinoid tumors. *J Chromatogr B.* 2020;1158:122395.

27. Hickman PE, Koerbin G, Potter JM, Abhayaratna WP. Statistical considerations for determining high-sensitivity cardiac troponin reference intervals. *Clin Biochem.* 2017;50:502-505.

28. Wang S, Ho V, Roquemore-Goins A, Smith FA. Effects of blood collection tubes, including pediatric devices, on 16 common immunoassays. *Clin Chem.* 2006;5:892-893.

29. Theiler-Schwetz V, Zaufel A, Schlager H, Obermayer-Pietsch B, Fickert P, Zollner G. Bile acids and glucocorticoid metabolism in health and disease. *Biochim Biophys Acta Mol Basis Dis.* 2019;1865:243-251.

30. de Aguiar Vallim TQ, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. *Cell Metab.* 2013;17:657-669.

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