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Differentiation of various traditional Chinese medicines derived from animal bile and gallstone: Simultaneous determination of bile acids by liquid chromatography coupled with triple quadrupole mass spectrometry

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ABSTRACT

Animal biles and gallstones are popularly used in traditional Chinese medicines, and bile acids are their major bioactive constituents. Some of these medicines, like cow-bezoar, are very expensive, and may be adulterated or even replaced by less expensive but similar species. Due to poor ultraviolet absorbance and structural similarity of bile acids, effective technology for species differentiation and quality control of bile-based Chinese medicines is still lacking. In this study, a rapid and reliable method was established for the simultaneous qualitative and quantitative analysis of 18 bile acids, including 6 free steroids (cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, hyodeoxycholic acid, and ursodeoxycholic acid) and their corresponding glycine conjugates and taurine conjugates, by using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). This method was used to analyze six bile-based Chinese medicines: bear bile, cattle bile, pig bile, snake bile, cow-bezoar, and artificial cow-bezoar. Samples were separated on an Atlantis dC_{18} column and were eluted with methanol–acetonitrile–water containing ammonium acetate. The mass spectrometer was monitored in the negative electrospray ionization mode. Total ion currents of the samples were compared for species differentiation, and the contents of bile acids were determined by monitoring specific ion pairs in a selected reaction monitoring program. All 18 bile acids showed good linearity ($r^2 > 0.993$) in a wide dynamic range of up to 2000-fold, using dehydrocholic acid as the internal standard. Different animal biles could be explicitly distinguished by their major characteristic bile acids: tauroursodeoxycholic acid and taurochenodeoxycholic acid for bear bile, glycocholic acid, cholic acid and taurocholic acid for cattle bile, glycohyodeoxycholic acid and glycochenodeoxycholic acid for pig bile, and taurocholic acid for snake bile. Furthermore, cattle bile, cow-bezoar, and artificial cow-bezoar could be differentiated by the existence of hyodeoxycholic acid and the ratio of cholic acid to deoxycholic acid. This study provided bile acid profiles of bile-based Chinese medicines for the first time, which could be used for their quality control.

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1. Introduction

Bile acids (BAs) are a group of steroids bearing a carboxyl group at C-17 side chain, and represent the characteristic constituents of human and animal bile [1–3]. They play an important role in lipid absorption and cholesterol catabolism, and may be promising therapeutic agents to increase the intestine absorption of vitamins, to correct biliary cholesterol saturation, and to treat cholesterol gallstones and cholestatic liver diseases [1,4,5]. Ursodeoxycholic acid (UDCA) has been approved by Food and Drug Administration for clinical use of gallstone dissolution and prevention [6]. On the other hand, animal biles and gallstones, which contain high amounts of bile acids, have been used as traditional medicines for a long history in many countries including China, Japan, Korea, and India. Today, different species of biles are recorded in national pharmacopoeias [7–12] (Table 1S).

In traditional Chinese medicine (TCM), six bile-based crude drugs are widely used, including bear bile, cattle bile, pig bile, snake bile, cow-bezoar (naturally occurred), and artificial cow-bezoar. Although these drugs are derived from biles and gallstones, their therapeutic functions and target organs in the TCM theory are significantly different, as summarized in Table 1 [12,13]. For example, cow-bezoar is mainly used for serious emergency diseases like coma, stroke and convulsion, while snake and pig biles are ordinary medicines for cough and gastrointestinal diseases [13–15]. Market prices of these crude drugs are remarkably different as well. Due to limited natural resource, cow-bezoar, bear bile and snake bile are more expensive than cattle bile and pig bile. Natural cow-bezoar, given its incomparable therapeutic effects, is one of the most precious Chinese medicines, and is at least 1000-fold more

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Table 1
Medicinal animal biles and corresponding formulated pharmaceutical products used in this study.

Generic name	Formal Latin name	Natural source	Code	Actions in TCM	Target organs in TCM	Pharmaceutical products	Code	Dosage form
Cow-bezoar	Calculus Bovis	Cattle	CB	To restore consciousness, eliminate *phlegm*, relieve convulsion, and counteract *toxicity*	Liver	Angong Niuhuang Pills	CB-P	Big honeyed pill
Artificial cow-bezoar	Calculus Bovis Artifactus	Cattle	CBA	To clear *heat* and *toxin*, eliminate *phlegm* and relieve convulsion	Liver	Niuhuang Jiedu Tablets	CBA-P	Tablet
Cattle bile	Fel Bovis	Cattle	FB	To counteract *toxicity*, and relieve swelling	Eye, gut, liver, gallbladder			
Snake bile	Fel Serpentis	Snake	FS	To dispel *wind* and *damp*, relieve coughing, eliminate *phlegm*, and counteract *toxicity*	Liver, lung, eye	Shedan Chuanbei Liquid	FS-P	Oral liquid
Pig bile	Pulvis Fellis Suis	Pig	PFS	To clear *heat* and *toxin*, moisten *dry*, and relieve cough and asthma	Lung, stomach, gallbladder	Huodan Pills	PFS-P	Water-honeyed pill
Bear bile	Pulvis Fellis Ursi	Bear	PFU	To clear *heat* and *toxin*, soothe pain, sedate, and counteract bacteria	Eye, liver, stomach	Xiongdan Huangqin Drops	PFU-P	Eye drop

Note: TCM, traditional Chinese medicine.

expensive than cattle bile. Artificial cow-bezoar has been developed as a substitute by mixing cattle bile, cholic acid, hyodeoxycholic acid, taurine, bilirubin and cholesterol to mimic natural cow-bezoar [12,13]. However, artificial cow-bezoar is much less expensive than the natural form, and is generally considered less effective. Bear bile and snake bile were approximately 200-fold more expensive than cattle bile and pig bile. Due to the significant difference in price, cattle bile and pig bile have been frequently reported to adulterate cow-bezoar or bear bile in China's natural medicine market. To be even worse, cattle bile and pig bile were used to substitute cow-bezoar in formulated Chinese medicines for emergency use [16–18]. These adulterants or counterfeits significantly compromised the therapeutic effects of Chinese medicines. Therefore, analytical technologies are needed to differentiate and identify these bile species to guarantee their quality and efficacy.

A number of methods have been developed to determine bile acids in Chinese medicines. However, only a few BAs were analyzed in these studies [7,14,15,19,20]. No report is available to systematically clarify the chemical constituents of various bile-based Chinese medicines, and to find out diagnostic differences for their identification and quality control. Furthermore, the concurrent existence, similar structures, and poor ultraviolet absorbance of BAs rendered their separation and identification problematic. Current approaches were mainly based on high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography (GC) [3,15,21], using evaporative light scattering detector (ELSD) [14,19] or mass spectrometry (MS) as detector. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) could be the most sensitive and reliable technology for the analysis of bile acids [3]. HPLC provided effective chromatographic separation, while MS could efficiently ionize bile acids, especially in the negative electrospray ionization (ESI) mode, to produce diagnostic fragment ions by tandem mass spectrometry. Moreover, LC/MS/MS could provide high sensitivity and wide dynamic range for quantitative analysis when it was operated in the selected reaction monitoring (SRM) mode on a triple quadrupole mass spectrometer.

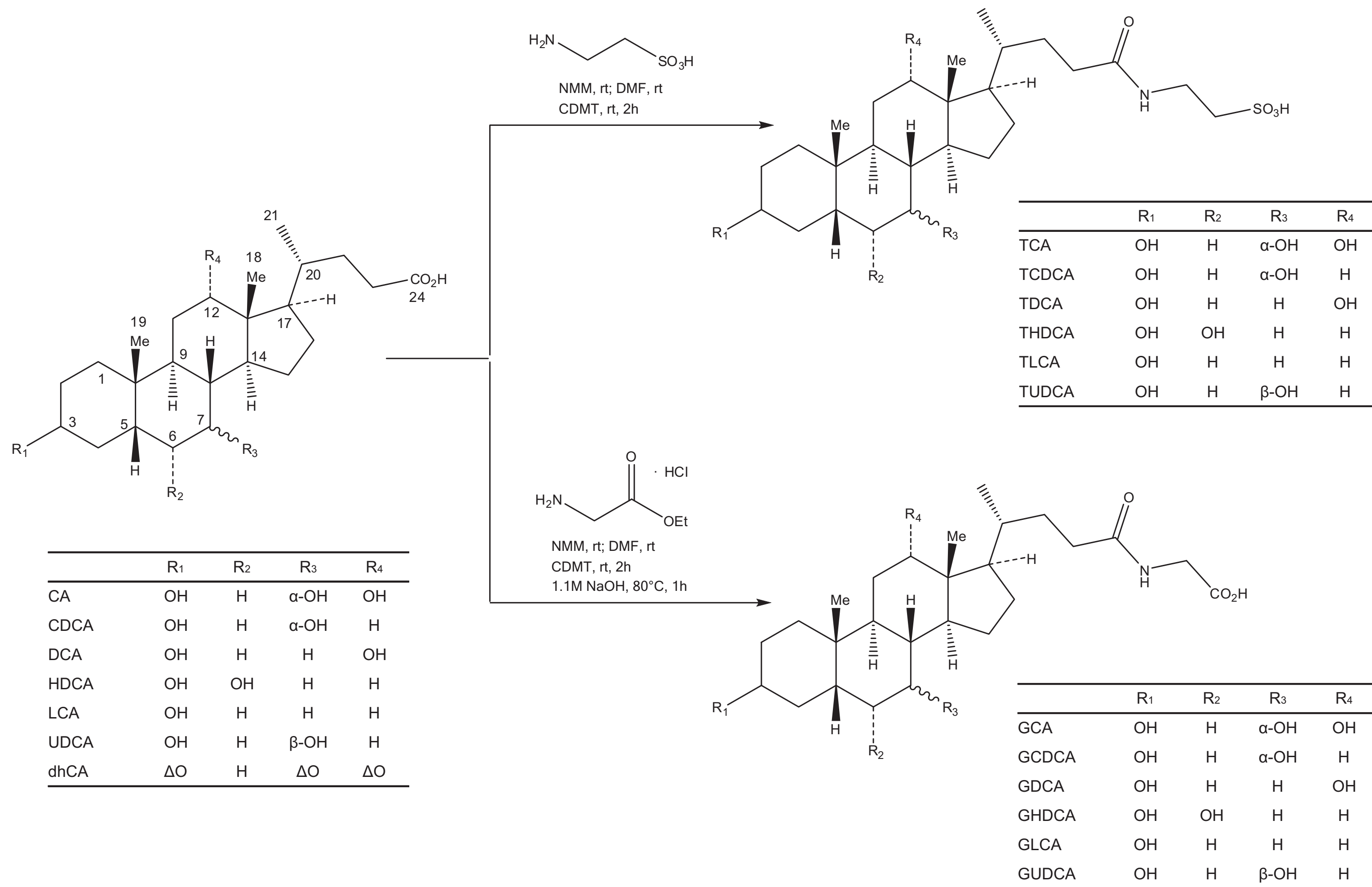
In this study, a rapid and sensitive LC/MS/MS method was established to simultaneously determine 18 BAs in bile-based crude drugs both qualitatively and quantitatively. Chemical differences among six crude TCM drugs were elucidated for the first time, and could be used for the quality control of both raw materials and formulated products.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, methanol (J.T. Baker, NJ, USA), ammonium acetate, and ammonium hydroxide (Sigma–Aldrich, MO, USA) were of HPLC grade. De-ionized water was prepared by a Milli-Q system (Millipore, MA, USA). High-purity nitrogen (99.9%) and helium (99.99%) were from Gas Supply Center of Peking University Health Science Center (Beijing, China).

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and taurochenodeoxycholic acid (TCDCA) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glycochenodeoxycholic acid (GCDCA) and lithocholic acid (LCA) were from Sigma–Aldrich (MO, USA). Hyodeoxycholic acid (HDCA) and dehydrocholic acid (dhCA) were from Tianqi Chemical Engineering (Anhui, China). UDCA was from Bio Basic Inc. (Ontario, Canada). The other BAs were synthesized by the authors. Crude drugs (Table 2S) and corresponding formulated products (Table 3S) were purchased from pharmacies around China, and were identified by



	R1	R2	R3	R4
CA	OH	H	α-OH	OH
CDCA	OH	H	α-OH	H
DCA	OH	H	H	OH
HDCA	OH	OH	H	H
LCA	OH	H	H	H
UDCA	OH	H	β-OH	H
dhCA	ΔO	H	ΔO	ΔO

	R1	R2	R3	R4
TCA	OH	H	α-OH	OH
TCDCA	OH	H	α-OH	H
TDCA	OH	H	H	OH
THDCA	OH	OH	H	H
TLCA	OH	H	H	H
TUDCA	OH	H	β-OH	H

	R1	R2	R3	R4
GCA	OH	H	α-OH	OH
GCDCA	OH	H	α-OH	H
GDCA	OH	H	H	OH
GHDCA	OH	OH	H	H
GLCA	OH	H	H	H
GUDCA	OH	H	β-OH	H

Fig. 1. Chemical synthesis of conjugated bile acids.

Dr. Min Ye. Voucher specimens were deposited at the authors' laboratory.

2.2. Synthesis and characterization of conjugated bile acids

The methods described by Willemen et al. [22,23] were modified to synthesize conjugated bile acids from corresponding free bile acids (CA, DCA, HDCA, LCA, UDCA), as depicted in Fig. 1. The synthesized reference compounds were taurine conjugates, including taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurohyodeoxycholic acid (THDCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA); and glycine conjugates, including glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycohyodeoxycholic acid (GHDCA), glycolithocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA). The method described by Hoogwater and Peereboom [24] was applied to synthesize ethyl aminoacetate hydrochloride. The reaction products were respectively applied to silica gel and Sephadex LH-20 columns to obtain pure compounds. The structures were identified based on their ^{1}H NMR (Fig. 1S), ^{13}C NMR (Fig. 2S–7S) and mass spectra, and by comparing with literature data [25]. Purities of the synthesized compounds were above 98%, suggesting these compounds could be used as reference standards for chemical analysis. Their structures are shown in Fig. 1.

2.3. Preparation of stock solutions and calibration standard solutions

CDCA (2500 nmol), CA (2420 nmol), DCA (3800 nmol), HDCA (3240 nmol), LCA (3400 nmol), UDCA (3350 nmol), GCDCA (2690 nmol), GCA (2080 nmol), GDCA (2310 nmol), GHDCA (2090 nmol), GLCA (2470 nmol), GUDCA (2340 nmol), TCDCA (2160 nmol), TCA (2320 nmol), TDCA (2540 nmol), THDCA (1920 nmol), TLCA (2420 nmol) and TUDCA (2470 nmol) were dissolved separately in 1 mL of methanol to prepare their individual stock solutions. These stock solutions were mixed and then serially diluted (dilution factor = 1.00, 1.67, 5.00, 16.67, 50.00, 166.70, 500, 1667, and 5000) to produce calibration standard solutions. Compound dhCA was used as the internal standard (IS), and was dissolved in methanol to produce a spiking solution (2.026 nmol/mL). Calibration standard samples were produced by mixing each calibration standard solution with IS spiking solution (1:1, v/v). Quality control samples (QC samples) were prepared in the same procedure at three concentration levels (Table 4S). All the solutions were sealed and stored at −20 °C until use, and were kept at 15 °C during analysis.

2.4. Sample preparation

All crude drugs and pharmaceutical products were stored in vacuum desiccators until use. A full list is given in Table 1. For sample preparation, all crude drugs were pulverized into fine powders, and an aliquot of 3 mg was used except for FS-1 (9 mg) and FS-2 (18 mg). For solid formulated products (CBA-P, CB-P, PFS-P), the samples were pulverized into fine powders, and an aliquot of 2.0 g was used. For liquid formulated products (FS-P, PFU-P), a 2.0-mL aliquot was used. Each sample was dissolved in 20 mL of methanol and 20 mL of IS solution for quantitative analysis or in 40 mL of methanol for qualitative analysis. The solution was vortexed at 2000 rpm for 2 min, ultrasonicated for 20 min (40 kHz, 300 W), and vortexed (2000 rpm) again for 2 min. The supernatants were filtered through 0.22 μm membranes before use. A 5-μL aliquot was injected for LC/MS analysis.

Table 2
MS/MS detection parameters and calibration curves of 18 bile acids with dhCA as the internal standard.

Analyte	$[M-H]^-$ (m/z)	SRM transitions	Collision energy (V)	Tube lens offset (V)	Regression equations	r^2	Linear range (nmol/mL)	LOD (nmol/mL)
CA	407.3	407.3 → 289.3	50	−190	$y = 0.4648x - 0.1659$	0.9960	0.364–60.619	0.012
CDCA	391.3	391.3 → 391.3	17	−197	$y = 10.1225x + 0.4009$	0.9965	0.125–62.453	0.012
DCA	391.3	391.3 → 391.3	17	−197	$y = 21.8634x + 0.3876$	0.9959	0.057–94.955	0.019
GCA	464.3	464.3 → 74.0	58	−164	$y = 0.1392x - 0.0412$	0.9961	0.313–52.116	0.104
GCDCA	448.3	448.3 → 74.0	51	−164	$y = 0.9121x - 0.0971$	0.9931	0.134–67.222	0.013
GDCA	448.3	448.3 → 74.0	51	−164	$y = 0.1834x - 0.0631$	0.9949	0.347–57.866	0.116
GHDCA	448.3	448.3 → 74.0	51	−164	$y = 0.2920x - 0.0874$	0.9969	0.314–52.302	0.031
GLCA	432.3	432.3 → 388.3	46	−162	$y = 0.2453x - 0.0278$	0.9970	0.123–61.733	0.012
GUDCA	448.3	448.3 → 74.0	51	−164	$y = 0.5304x - 0.0736$	0.9978	0.351–58.422	0.035
HDCA	391.3	391.3 → 391.3	17	−197	$y = 11.3262x + 0.5556$	0.9959	0.049–48.561	0.016
LCA	375.2	375.2 → 375.2	18	−175	$y = 22.1972x - 0.0687$	0.9971	0.051–51.023	0.017
TCA	514.3	514.3 → 80.0	58	−200	$y = 0.1930x - 0.0208$	0.9967	0.116–58.054	0.035
TCDCA	498.3	498.3 → 124.0	57	−207	$y = 0.5696x - 0.0508$	0.9978	0.108–54.076	0.032
TDCA	498.3	498.3 → 124.0	57	−207	$y = 0.3267x - 0.0306$	0.9961	0.127–63.590	0.038
THDCA	498.3	498.3 → 124.0	57	−207	$y = 0.3340x - 0.0416$	0.9961	0.288–48.068	0.029
TLCA	482.3	482.3 → 80.0	56	−203	$y = 0.1772x - 0.0187$	0.9971	0.121–60.521	0.036
TUDCA	498.3	498.3 → 124.0	57	−207	$y = 0.4945x - 0.0460$	0.9982	0.123–61.747	0.037
UDCA	391.3	391.3 → 391.3	17	−197	$y = 11.0256x + 0.0444$	0.9953	0.050–50.281	0.017
dhCA	401.3	401.3 → 249.3	39	−137	–	–	–	–

Note: In the regression equation $y = ax + b$, x refers to the concentration of bile acid analytes (nmol/L); y the ratio of analyte peak area/IS peak area; and r the correlation coefficient. LOD, limit of detection.

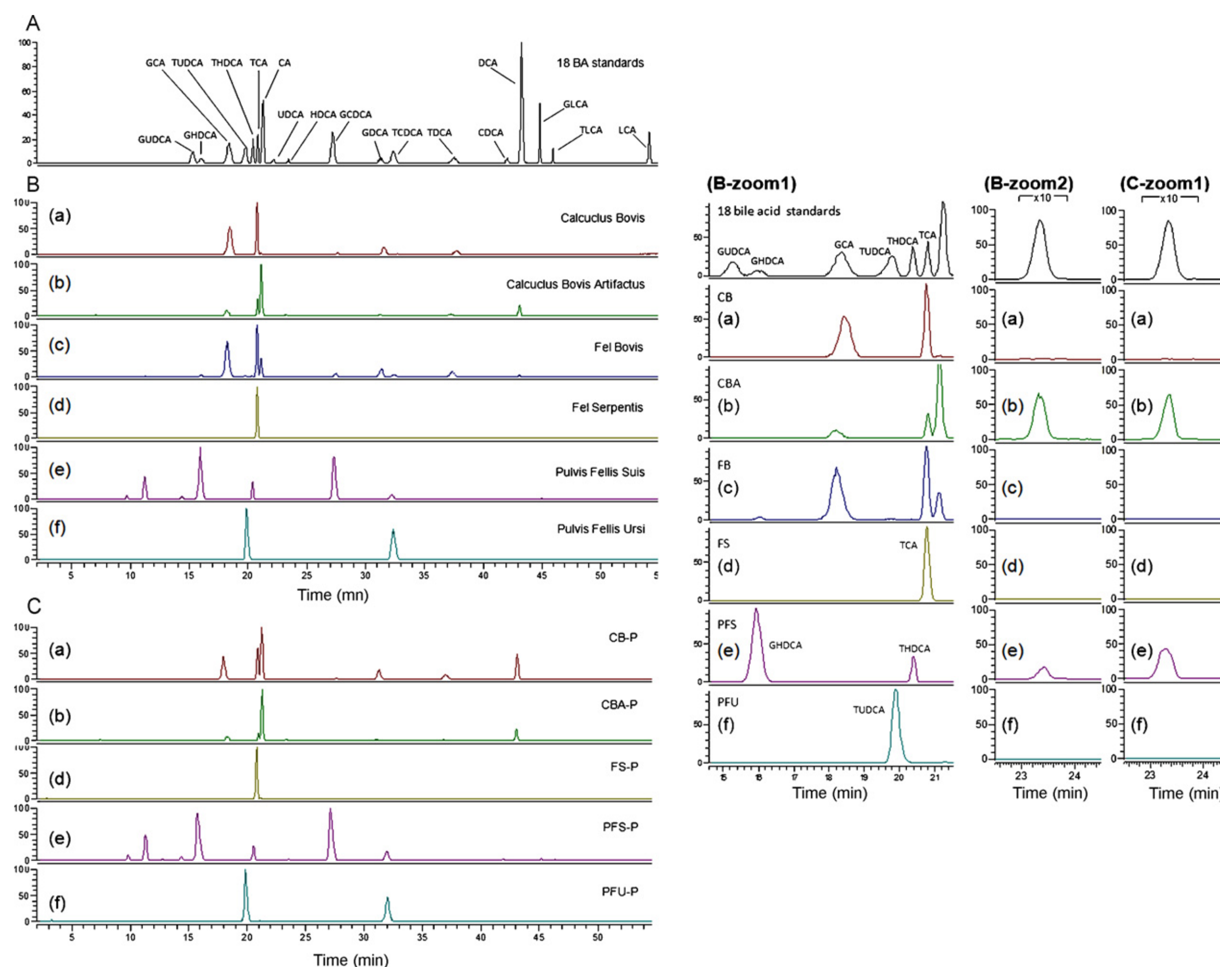


Fig. 2. LC/MS chromatograms of bile-based crude drugs and formulations. (A) Total ion current of a standard solution containing 18 BAs, (B) total ion currents of bile-based crude drugs, and (C) extracted ion chromatograms of pharmaceutical products containing bile-based crude drugs: (B-zoom 1) zoom 14.5–21.5 min of (B); (B-zoom 2) zoom 22.5–24.5 min of (B); (C-zoom 1) zoom 22.5–24.5 min of (C).

2.5. LC/MS/MS conditions

The LC/MS/MS system consisted of a Finnigan Surveyor LC instrument connected to a Finnigan TSQ Quantum triple quadrupole mass spectrometer via ESI interface (ThermoFisher, CA, USA). The mobile phase consisted of acetonitrile (A), methanol (B), and water containing ammonium acetate (C). For qualitative analysis, an Atlantis dC_{18} column (5 μm, ID 4.6 mm × 250 mm) equipped with an XTerra MS C_{18} guard column (5 μm, ID 3.9 mm × 20 mm) (Waters, MA, USA) was used. The mobile phase (C) was 4 mM ammonium acetate in water. Gradient elution program, 0 min, 27:2:71; 13 min, 28:2:70; 15–35 min, 32:0:68; 45 min, 55:0:45; 55 min, 85:0:15 (A:B:C, v/v/v). Flow rate, 1.0 mL/min. Post-column splitting ratio, 4:1. For quantitative analysis, an Atlantis dC_{18} column (5 μm, ID 3.9 mm × 150 mm) equipped with an XTerra MS C_{18} guard column (5 μm, ID 3.9 mm × 20 mm) was used. The mobile phase (C) was 10 mM ammonium acetate in water (adjusted to pH 8 with ammonium hydroxide). Gradient elution program, 0–5 min, 22:8:70; 30 min, 30:10:60; 45 min, 60:20:20 (A:B:C, v/v/v). Flow rate, 0.5 mL/min. The HPLC effluent was introduced into the mass spectrometer without splitting. The column temperature was set to 20 °C, and the sample tray temperature was maintained at 15 °C.

For MS detection, the ESI source was operated in the negative ion mode. High purity nitrogen was used as the sheath (50 arb) and auxiliary (10 arb) gas; high purity argon was used as the collision gas (1.0 mTorr). Parameters were as follows: spray voltage, 4.0 kV; capillary temperature, 350 °C; capillary offset, −35 V; source-fragmentation voltage, 10 V. Qualitative analyses were performed in the full scan mode (*m*/*z* 150–800), while quantitative analyses were monitored in SRM mode. The SRM ion pair transitions and collision energy levels are listed in Table 2. Q1 and Q3 quadrupoles were set at unit resolution.

3. Results and discussion

3.1. Chromatographic separation of 18 bile acids

For sample extraction, different solvents (water, methanol, methanol–water, and chloroform) and different extraction methods (ultrasonic bath and maceration) were compared. Methanol showed better extraction efficiency than other solvents. Ultrasonic bath was more efficient than maceration, especially for honeyed pills. Therefore, all samples were extracted with methanol in ultrasonic bath in this study, unless otherwise stated.

Due to similar physico-chemical properties of bile acids, desirable separation was difficult to obtain. Different types of HPLC C_{18} columns were tested (Atlantis dC_{18}, Waters; Luna C_{18}, Phenomenex; Platinum C_{18}, Alltech; XTerra, Waters; YMC ODS-A, YMC; Zorbax SB-C_{18}, Agilent), and Atlantis dC_{18} column was selected (Fig. 8S).

The HPLC mobile phase was optimized to separate bile acids. A number of solvent combinations were tested (acetonitrile–water, methanol–water, and methanol–acetonitrile–water). Finally, a three-component solvent system, methanol–acetonitrile–aqueous

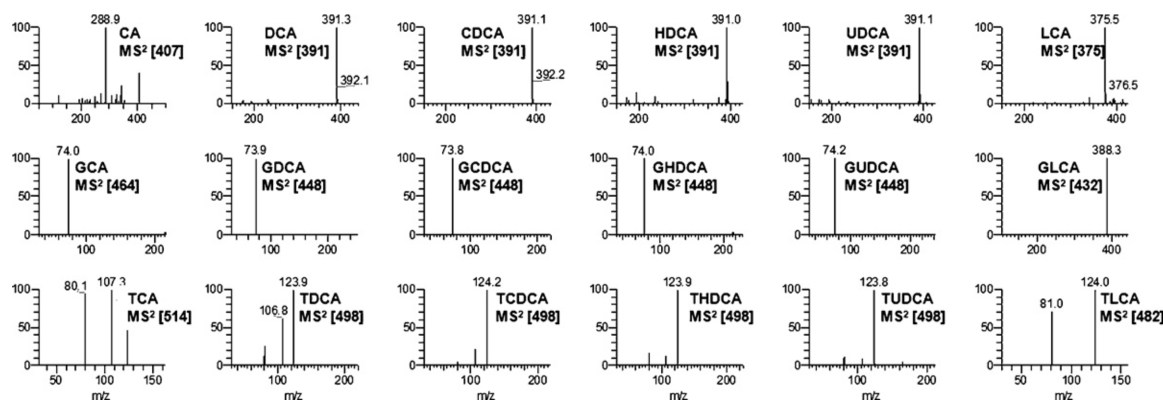


Fig. 3. Electrospray ionization-tandem mass spectra of 18 bile acids in the negative ion mode.

buffer, was found to give the best chromatographic resolution. This result was in agreement with previous reports [3,26,27]. Then different modifiers in water were tried, including acidic additives (formic acid, 0.01%, 0.1%) and alkaline additives (ammonium formate, ammonium acetate, triethylamine, 2 mM). Alkaline modifiers, especially ammonium acetate, not only improved chromatographic separation and peak shape, but also remarkably increased MS detection sensitivity. Acid modifiers, though improved MS response, exhibited little benefit for chromatographic resolution. In addition, different concentrations of ammonium acetate (2 mM, 4 mM, 5 mM, 10 mM) were tested, and 4 mM was selected for the qualitative method and 10 mM for the quantitative method, based on chromatographic separation, MS sensitivity, and MS repeatability (Fig. 9S). Furthermore, the column temperature (15 °C, 20 °C, 30 °C, 40 °C, 50 °C) and flow rate (1.0 mL/min, 0.8 mL/min) were also optimized, and a flow rate of 1.0 mL/min at 20 °C were finally used. Total ion current (TIC) of the 18 BA standards is shown in Fig. 2A. The above HPLC conditions were used for quantitative LC/MS/MS analyses after minor modifications.

3.2. Optimization of MS conditions

BAs could efficiently produce deprotonated molecular ions in ESI source [27]. Solutions of 18 pure BAs and the internal standard (0.1 mg/mL in methanol mixed with an equal volume of the mobile phase) were individually injected into the ESI source by continuous infusion in negative ion mode to give MS/MS spectra (Fig. 3A). Similar to previous reports [27,28], conjugated BAs exhibited typical fragment ions of glycine (*m*/*z* 74) or taurine (*m*/*z* 80 or 124) moiety, which were used for SRM ion pairs (Table 2). For unconjugated BAs, however, prominent product ions were not usually observed [3,27,28]. Only two analytes CA (407.3 → 289.3) and dhCA (401.3 → 249.3) produced significant fragment ions for SRM detection (Fig. 3A). For the other unconjugated BAs, the $[M-H]^-$ ions were used as both precursor and product ions for their SRM transitions [27,28]. Collision energy and tube lens offset were optimized for all ion pairs, as shown in Table 2. A typical SRM chromatogram of 18 analytes is given in Fig. 5.

3.3. Validation of the LC/MS/MS quantitation method

All data were processed with Xcalibur 2.0.7 software (ThermoFisher, CA, USA). Quantification was performed using dhCA as the internal standard, which was absent in both the crude drugs and their formulated pharmaceutical products (Fig. 4). Specificity of this method was evaluated by analyzing an artificial matrix, which was prepared following the recipe of PFS-P but without pig bile. None of the 18 bile acids were detected in this matrix (Fig. 10S). All 18 analytes showed good linearity ($r^2 > 0.993$) in a wide dynamic range

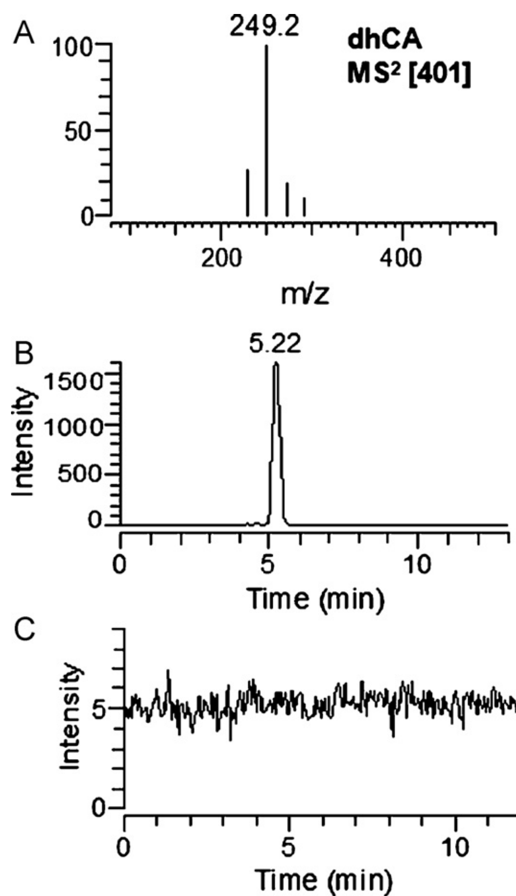


Fig. 4. MS/MS spectra and SRM chromatograms of dehydrocholic acid to show specificity of the LC/MS/MS method. (A) MS/MS spectra of *m*/*z* 401, (B) SRM chromatogram (401.3 → 249.3) of IS spiked sample, and (C) SRM chromatogram (401.3 → 249.3) of crude drug FB-1 (without internal standard spiking). SRM, selected reaction monitoring.

of 160–2000-fold (0.049–0.364 to 48.068–94.955 nmol/mL). The precision was determined by calculating the relative standard deviation (RSD) of peak areas at five concentration levels measured in the same day ($n = 5$) and in five consecutive days. Following the recommendations for bioanalytical method validation by the Food and Drug Administration [29], five concentration levels covering the dynamic range of the calibration curve were examined. ULQC, LQC, MQC, HQC, and UHQC represented ultra-low, low, middle, high, and ultra-high concentrations, respectively. As shown in Table 3, the RSD values were below 16.12%, indicating acceptable precision of the method. Meanwhile, the accuracy ranged from 85.56

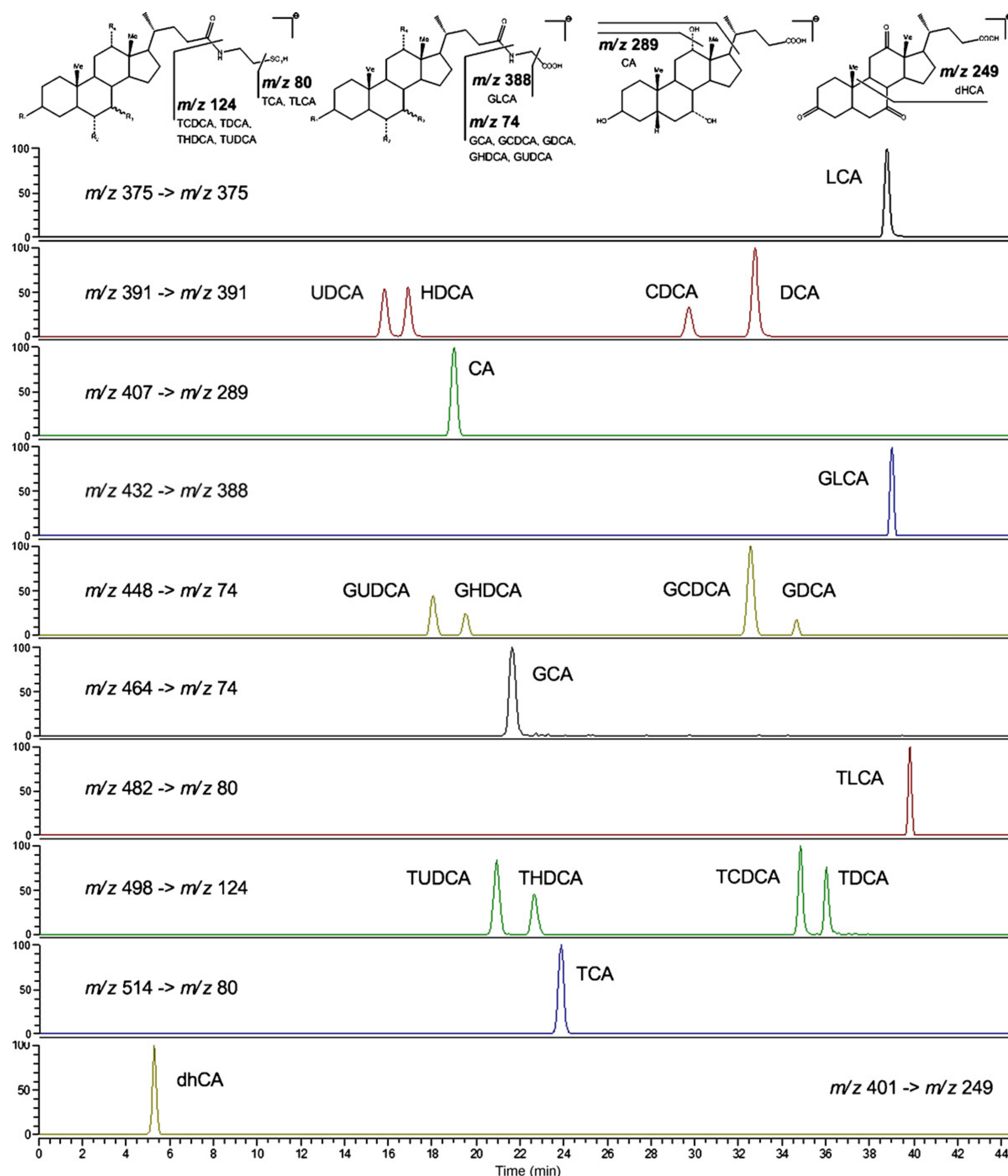


Fig. 5. Typical SRM chromatograms and (−)-ESI-MS/MS fragmentation pathways of 18 BAs. SRM, selected reaction monitoring; ESI, electrospray ionization; dhCA, dehydrocholic acid as the internal standard.

to 114.12% for the 18 analytes. In order to determine the recoveries of bile acids during sample pretreatment, a standard addition test was conducted at three concentration levels. A 4-fold diluted sample CBA-1 for qualitative analysis was used as the matrix. The 18 bile acids at three known concentrations were added into the matrix, and went through all the sample treatment steps described under Section 2.4. Recoveries were calculated by the formula: recovery (%) = concentration found/concentration spiked × 100%. Recoveries of the 18 bile acids ranged from 81.88 to 113.57% (Table 4S). Limits of detection (LOD) were measured by injecting serially diluted calibration standard solutions and calculated according to signal/noise = 3. The LOD ranged 0.012–0.116 nmol/mL for the 18 analytes (Table 2). In addition, all samples were kept in the sample tray at 15 °C during the tests. Stabilities of the bile acids were proved acceptable (RSD < 9.2%) over 120 h (Table 5S).

3.4. LC/MS fingerprints of bile acids in crude drugs and formulated products

The LC/MS TIC fingerprints of bile-based crude drugs and corresponding formulated products are shown in Fig. 2. By comparing with 18 pure standards and by analyzing their tandem mass spectra, major bile acids in the chromatograms were characterized (Fig. 3).

The bile acid fingerprints could be used to distinguish different crude drugs, and to identify the species according to their characteristic bile acids, as shown in Fig. 2B. Pig bile contained noticeable amounts of GHDCA, THDCA, GCDCA, and HDCA (see Fig. 2, B-zoom

Table 3
Intra- and interday precision, and accuracy of bile acids ($n = 5$).

Analytes	CA	CDCA	DCA	GCA	GCDCA	GDCA	GHDCA	GLCA	GUDCA	HDCA	LCA	TCA	TCDCA	TDCA	THDCA	TLCA	TUDCA	UDCA
ULQC (nmol/mL)																		
Intraday																		
VE	0.364	0.375	0.570	0.313	0.403	0.347	0.314	0.370	0.351	0.486	0.510	0.348	0.324	0.382	0.288	0.363	0.370	0.503
VO	0.389	0.376	0.603	0.327	0.400	0.370	0.334	0.360	0.362	0.455	0.526	0.347	0.314	0.374	0.267	0.368	0.400	0.495
RSD	2.74	9.71	6.96	6.33	4.19	2.62	6.55	8.54	10.91	8.08	4.45	4.45	11.80	7.97	6.45	10.99	4.01	8.54
A	106.84	100.19	105.85	104.36	99.18	106.65	106.24	97.30	103.19	93.72	103.20	99.79	96.92	97.89	92.70	101.25	108.22	98.39
Interday																		
VO	0.390	0.372	0.575	0.357	0.426	0.385	0.337	0.361	0.334	0.452	0.487	0.338	0.317	0.363	0.279	0.356	0.378	0.467
RSD	1.33	11.72	8.08	16.12	7.75	4.84	7.47	12.97	9.94	9.40	10.60	8.74	12.16	6.79	9.95	9.15	8.26	10.72
A	107.01	99.11	100.81	114.12	105.71	111.08	107.26	97.48	95.21	92.97	95.49	97.15	97.95	95.13	96.84	97.96	102.16	92.77
LQC (nmol/mL)																		
Intraday																		
VE	1.421	0.873	0.887	0.497	0.728	0.985	0.534	1.124	0.861	1.354	0.902	1.392	0.776	0.887	0.972	1.082	0.882	1.420
VO	1.400	0.880	0.940	0.494	0.766	0.953	0.552	1.113	0.924	1.401	0.918	1.356	0.716	0.903	0.981	1.018	0.872	1.414
RSD	8.37	8.38	4.57	3.82	4.88	5.80	1.71	11.82	1.60	5.39	4.83	6.27	7.43	6.09	7.49	6.41	10.36	4.34
A	98.53	100.84	105.93	99.39	105.26	96.75	103.28	99.01	107.32	103.49	101.81	97.40	92.24	101.82	100.92	94.08	98.82	99.55
Interday																		
VO	1.389	0.908	0.926	0.540	0.739	0.959	0.534	1.129	0.831	1.434	0.927	1.358	0.777	0.849	0.977	1.077	0.923	1.462
RSD	6.00	9.27	5.74	8.70	6.27	4.25	6.44	6.58	7.64	3.62	6.07	7.80	7.20	9.41	6.59	8.30	6.93	2.92
A	97.75	104.00	104.34	108.64	101.53	97.36	99.98	100.48	96.52	105.89	102.81	97.57	100.15	95.75	100.50	99.51	104.69	102.95
MQC (nmol/mL)																		
Intraday																		
VE	7.105	4.365	4.435	2.486	3.640	4.925	2.670	5.620	4.305	6.770	4.510	6.960	3.880	4.435	4.860	5.410	4.410	7.100
VO	6.902	4.164	4.291	2.425	3.508	4.837	2.556	5.800	4.314	6.881	4.399	7.403	4.151	4.595	4.758	5.128	4.324	7.064
RSD	3.94	7.64	7.49	3.43	7.00	9.35	3.76	8.05	6.19	8.09	6.15	3.40	2.67	4.66	5.86	3.25	6.91	4.56
A	97.14	95.40	96.76	97.56	96.38	98.22	95.74	103.21	100.20	101.63	97.54	106.36	106.98	103.60	97.91	94.78	98.05	99.49
Interday																		
VO	7.291	4.609	4.401	2.266	3.669	4.637	2.774	5.579	4.540	6.386	4.495	6.910	3.904	4.549	4.782	5.430	4.566	6.905
RSD	5.78	5.96	5.86	8.87	8.87	8.85	7.52	5.54	7.75	8.10	6.87	6.45	6.35	4.92	3.10	7.73	5.53	3.97
A	102.62	105.59	99.24	91.16	100.80	94.15	103.88	99.26	105.46	94.33	99.66	99.28	100.62	102.56	98.39	100.37	103.54	97.25
HQC (nmol/mL)																		
Intraday																		
VE	35.525	21.825	22.175	12.432	18.200	24.625	13.350	28.100	21.525	33.850	22.550	34.832	19.400	22.175	24.300	27.050	22.050	35.500
VO	32.147	21.972	22.594	12.336	18.328	23.883	13.800	30.016	21.870	33.500	23.581	34.866	19.726	21.909	23.708	27.511	23.717	34.621
RSD	3.94	3.76	6.51	2.60	8.06	5.87	6.74	2.06	2.84	6.17	6.28	6.27	7.45	4.91	3.33	3.52	5.28	4.88
A	90.49	100.67	101.89	99.22	100.70	96.99	103.37	106.82	101.60	98.97	104.57	100.10	101.68	98.80	97.56	101.70	107.56	97.52
Interday																		
VO	37.235	22.911	24.112	12.445	18.680	25.170	13.383	27.950	22.767	35.777	21.987	36.476	18.992	22.756	25.724	27.812	22.834	35.630
RSD	9.78	6.98	10.86	1.62	4.31	6.98	5.47	9.46	5.03	6.26	5.57	8.45	6.27	7.93	6.01	8.42	8.25	6.14
A	104.81	104.98	108.74	100.11	102.64	102.21	100.25	99.47	105.77	105.69	97.50	104.72	97.90	102.62	105.86	102.82	103.56	100.36
UHQC (nmol/mL)																		
Intraday																		
VE	36.371	37.472	56.973	31.270	40.333	34.720	31.381	37.040	35.053	48.561	51.023	34.800	32.446	38.154	28.841	36.313	37.048	50.281
VO	38.719	37.032	56.714	33.292	44.336	35.871	31.218	36.574	36.831	41.551	53.853	35.371	31.592	39.686	30.602	38.315	39.586	47.842
RSD	6.80	8.23	6.53	8.54	3.48	6.22	8.72	9.19	7.87	8.53	7.67	4.64	7.34	1.86	4.91	3.60	5.93	5.20
A	106.46	98.83	99.55	106.47	109.93	103.31	99.48	98.74	105.07	85.56	105.55	101.64	97.37	104.01	106.10	105.51	106.85	95.15
Interday																		
VO	38.550	37.382	57.991	32.076	43.135	33.896	31.015	38.104	37.872	43.967	52.472	36.538	33.261	39.726	28.482	38.086	40.160	48.182
RSD	7.21	8.06	7.98	11.36	8.00	12.20	12.59	6.22	9.12	5.53	11.43	5.68	12.84	4.03	6.62	8.34	5.65	6.33
A	105.99	99.76	101.79	102.58	106.95	97.63	98.83	102.87	108.04	90.54	102.84	104.99	102.51	104.12	98.75	104.88	108.40	95.83

Note: ULQC, LQC, MQC, HQC, and UHQC represent ultra-low, low, middle, high, and ultra-high concentration quality control samples, respectively. VE, value expected; VO, value observed; RSD, relative standard deviation; A, accuracy in %.

Table 4
Quantification of 18 bile acids in various medicinal biles (nmol/mg).

Sample	CA	CDCA	DCA	GCA	GCDCA	GDCA	GHDCA	GLCA	GUDCA	HDCA	LCA	TCA	TCDCA	TDCA	THDCA	TLCA	TUDCA	UDCA
CB-1	6.66	BL	4.75	110.76	BL	34.05	BL	ND	ND	ND	BL	68.99	1.86	24.89	ND	BL	ND	ND
CB-2	BL	ND	ND	279.65	3.05	100.26	BL	ND	ND	BL	ND	153.15	4.13	59.10	BL	BL	ND	BL
CB-3	12.55	BL	11.19	72.27	BL	17.15	BL	ND	ND	ND	BL	32.42	BL	15.16	ND	BL	ND	BL
CBA-1	102.65	8.18	31.63	27.08	BL	8.66	BL	ND	ND	30.08	BL	55.64	1.47	25.85	ND	BL	ND	BL
CBA-2	100.41	7.38	26.62	66.65	BL	19.45	BL	ND	ND	60.58	BL	82.89	1.46	23.82	ND	BL	ND	BL
CBA-3	65.38	6.61	59.18	124.11	2.82	62.07	BL	ND	ND	53.85	BL	105.34	3.07	43.33	BL	BL	ND	BL
FB-1	54.03	BL	6.61	587.51	19.47	174.73	36.39	ND	ND	BL	BL	249.76	20.69	131.73	BL	2.00	8.43	ND
FB-2	39.87	BL	6.39	536.96	7.12	148.12	BL	ND	ND	BL	BL	283.53	12.93	113.27	ND	2.72	ND	BL
FB-3	57.68	BL	7.44	634.76	18.97	199.14	37.22	BL	ND	BL	BL	364.75	26.13	145.38	4.64	3.28	9.66	BL
FS-1	BL	BL	BL	BL	BL	58.93	BL	BL	BL	BL	BL	1324.34	BL	44.72	BL	BL	BL	BL
FS-2	BL	BL	BL	BL	BL	10.42	BL	BL	BL	BL	BL	824.01	3.10	7.63	BL	BL	BL	BL
FS-3	BL	BL	BL	BL	BL	13.60	BL	BL	BL	BL	0.91	197.39	287.79	1.78	BL	BL	BL	BL
PFS-1	ND	7.55	BL	9.42	90.75	5.52	416.76	2.43	ND	11.15	BL	BL	20.66	ND	62.16	BL	BL	ND
PFS-2	ND	7.90	BL	9.91	83.81	4.84	310.84	1.66	ND	11.18	BL	ND	18.68	ND	47.04	1.63	ND	ND
PFS-3	4.84	6.00	BL	9.67	101.14	4.84	460.31	1.64	ND	10.31	BL	BL	21.23	ND	61.87	2.71	ND	ND
PFU-1	ND	2.02	BL	ND	BL	4.95	BL	ND	ND	BL	6.20	3.58	319.98	ND	ND	BL	358.61	0.68
PFU-2	ND	3.71	BL	ND	BL	4.77	ND	ND	ND	BL	ND	5.39	374.78	ND	ND	BL	518.81	1.30
PFU-3	4.93	36.37	BL	ND	BL	4.80	BL	ND	ND	BL	BL	2.04	178.49	ND	ND	BL	339.24	19.15

Note: The samples were coded following their abbreviations in Table 1. ND, not detected; BL, below the limit of quantification.

2) which were almost absent in all other natural biles or gallstones (except for artificial cow-bezoar). Particularly, HDCA and its derivatives, with a 6α-OH, could be considered as the characteristic constituents of pig bile. Bear bile contained high amounts of TUDCA and TCDCA. UDCA and its derivatives, with a 7β-OH, were characteristic constituents of bear bile, and were hardly detected in other natural species. Snake bile, however, contained TCA as the predominant constituent. Some of these results were in accordance with previous reports [5]. These characteristics could be used as chemical markers to identify bear bile, pig bile, and snake bile.

Bile products from cattle, including cow-bezoar, artificial cow-bezoar and cattle bile, however, showed more complicated bile acid profiles and were more difficult to be differentiated. By carefully analyzing their chemical profiles, we found that all the three species contained noticeable amounts of CA, GCA, TCA, DCA, GDCA, and TDCA, namely, derivatives of CA and DCA. Interestingly, both CA and DCA contained a 12α-OH. The simultaneous detection of the above six bile acids could be considered as chemical markers to distinguish cattle products from the other species.

The next step was to differentiate cattle bile, cow-bezoar, and artificial cow-bezoar. As these three species were all derived from cattle, their BA profiles showed high similarity. Fortunately, we managed to find out differences between these species. HDCA was a characteristic constituent of pig bile, which was used to manufacture artificial cow-bezoar. Thus, the detection of HDCA could be used to differentiate artificial cow-bezoar from cattle bile and natural cow-bezoar. Cattle bile contained significantly higher amounts of GCDCA, TCDCA and CA than cow-bezoar, and thus allowed their differentiation. It was interesting to note that the BA profile of natural cow-bezoar was relatively simple when compared to other bile-based crude drugs, although it is the most expensive one.

Previous studied reported that chemical composition of animal biles could be affected by many factors [30]. However, the chemical characteristics we discovered in this study were fairly conservative, which was demonstrated by analyzing three independent batches of each species. Therefore, these diagnostic characteristics could be used for their identification.

Furthermore, the above chemical characteristics were used to identify the bile-based crude drug ingredients in formulated Chinese medicine products. We analyzed the bile acids in five widely used commercial TCM products by LC/MS, each containing one species of bile-based crude drug (Table 1). Extracted ion chromatograms for 18 bile acids are given in Fig. 2C. Some of these preparations contain very complicated ingredients. For exam-

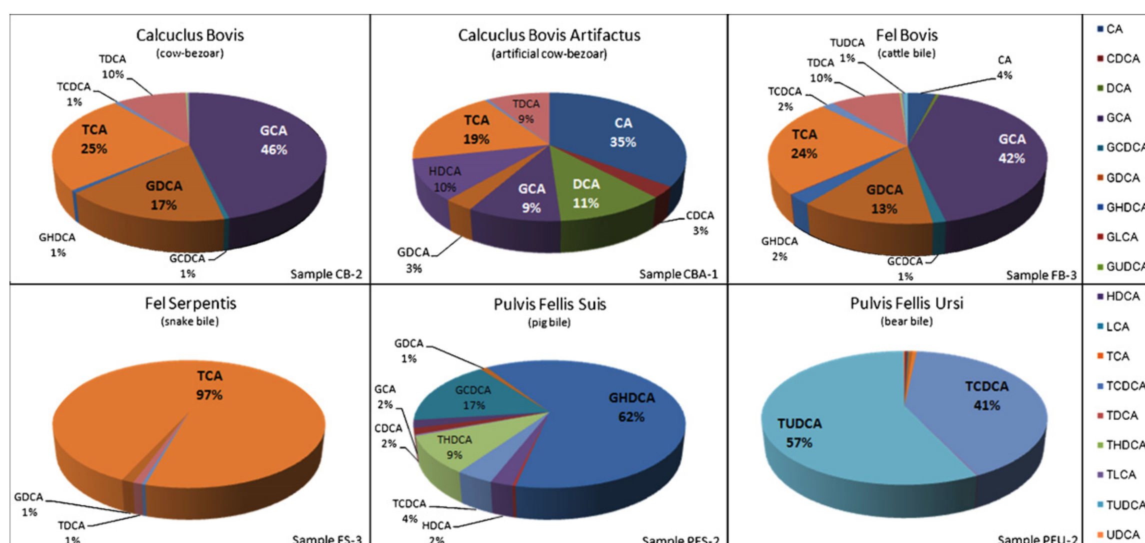


Fig. 6. Distribution of bile acids in various bile-based crude drugs.

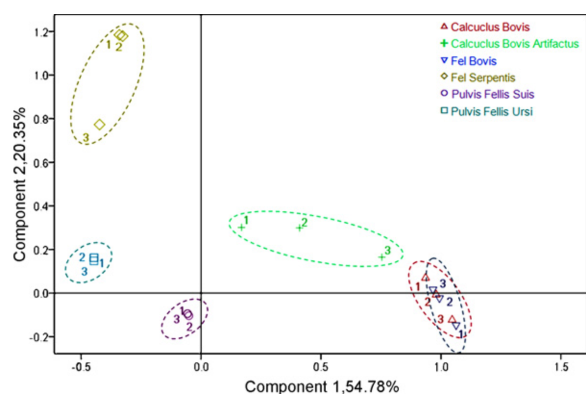


Fig. 7. Principal component analysis of bile acid composition in 18 batches of bile-based crude drugs.

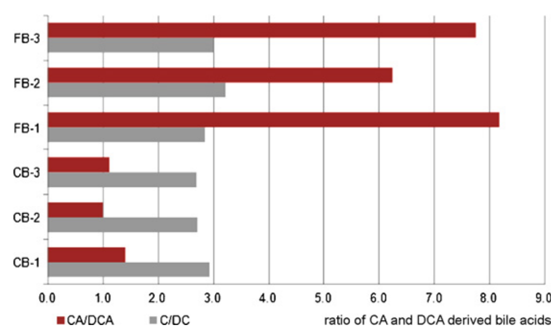


Fig. 8. Distribution of CA and DCA derivatives in cattle bile and natural cow-bezoar. CA/DCA, the amount ratio of CA/DCA; C/DC, the amount ratio of (CA + GCA + TCA)/(DCA + GDCA + TDCA). CA, cholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid.

ple, Angong Niuhuang Pills (CB-P) contained 11 ingredients, and Niuhuang Jiedu Tablets (CBA-P) contained 8 ingredients. These preparations also represented different dosage forms, including honeyed pills, tablets, oral liquids, and eye drops. Fortunately, bile acids in these preparations could be sensitively and specifically detected by LC/MS. More importantly, the bile-based crude drug ingredient in the preparations could be explicitly identified. As shown in Fig. 2C, the preparations showed bile acid profiles consistent to their corresponding crude drugs. The only exception was CB-P, which was supposed to use natural cow-bezoar. According to its bile acid profile, especially the relative ratio of TCA and CA, it was possible that both natural and artificial cow-bezoar were used in CB-P.

3.5. Distribution of bile acids in various animal biles and gallstones

Eighteen bile acids in six bile-based crude drugs were quantitatively determined by LC/MS/MS (Table 4). In accordance with LC/MS fingerprinting analysis, the relative contents of the bile acids varied significantly among the crude drugs (Fig. 6). HDCA and its conjugates are characteristic constituents of pig bile. They were all detected at medium to high levels, 10.3–11.1 nmol/mg of HDCA, 47.0–62.1 nmol/mg of THDCA, and 310–460 nmol/mg of GHDCA. GHDCA and GCDCA were the major bile acids in pig bile, and accounted for 80% of the total bile acids. TUDCA is the characteristic constituent of bear bile, and was detected at 339.2–518.8 nmol/mg levels. TCDCA was the other major constituent of bear bile, at 178.5–374.8 nmol/mg levels. TUDCA and TCDCA accounted for 95% of the total bile acids in three batches of bear bile. TCA was the only major constituent of snake bile, and was detected at 197.4–1324.3 nmol/mg levels. All cattle-derived crude drugs contained noticeable amounts of CA, GCA, TCA, GDCA, and TDCA. HDCA was only detected in artificial cow-bezoar (30.0–60.5 nmol/mg), and was not detected in natural cow-bezoar or cattle bile. Cattle bile contained remarkably higher amounts of TDCA and GCA than cow-bezoar, 563.9–634.7 nmol/mg versus 72.2–279.6 nmol/mg for GCA, and 113.2–145.3 nmol/mg versus 15.1–59.1 nmol/mg for TDCA. The quantitative data could be combined with the fingerprints to distinguish and characterize bile-based crude drugs.

The quantification data were further analyzed by principal component analysis (PCA). The covariance matrix calculated with PC1–PC2 rotated projection plot (promax, $\kappa = 50$) is shown in Fig. 7. The six drugs could be distinctively differentiated except cattle bile and natural cow-bezoar. These two species could be differentiated by the relative amounts of CA and DCA. As shown in Fig. 8, the CA/DCA ratio was around 1.2:1 for natural cow-bezoar, and around 7:1 for cattle bile, though the ratio of (CA + GCA + TCA)/(DCA + GDCA + TDCA) remained constant for these two species. Thus, the CA/DCA ratio may be a marker to different cattle bile and natural cow-bezoar.

The contents of individual bile acids varied significantly among three batches of each crude drug, with a relative standard deviation of 30–200%. This variation might be due to the complicated environments where biles and gallstones were formed. Interestingly, the relative ratios of unconjugated BAs, glycine-conjugated BAs, and taurine-conjugated BAs were relatively consistent among three batches for the same species (Fig. 9). However, the ratios varied remarkably for different species of crude drugs, and might also be used for their identification. Pig bile contained predominantly glycine-conjugated BAs (about 85%), while bear bile and snake bile mainly contained taurine-conjugated BAs (over 90%). On the other hand, all cattle-derived crude drugs contained noticeable amounts of both taurine-conjugated and glycine-conjugated BAs. Medium levels (around 30%) of unconjugated BAs were only detected in artificial cow-bezoar.

Although all animal biles and gallstones contained bile acids, these bile acids differed in structure and content. Slight changes in the structures of bile acids could significantly alter their biological activities. For instance, DCA could affect metabolic impairment, while UDCA showed a reverse function [31,32]. The difference in bile acid composition may be correlated with the therapeutic effects of bile-based crude drugs and their products. In the

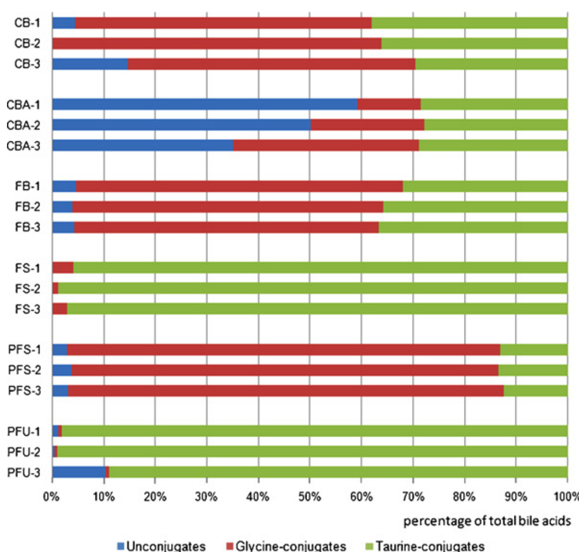


Fig. 9. Distribution of unconjugates, glycine-conjugates and taurine-conjugates of bile acids in bile-based crude drugs.

manufacturing of formulated TCM products, the replacement of expensive crude drugs like natural cow-bezoar and bear bile with cheap and readily available species like cattle bile and pig bile have been frequently reported [16–18]. Our results could be used to distinguish true and counterfeit products rapidly and accurately, and to guarantee the quality of bile-based Chinese medicines.

4. Conclusion

Animal biles and gallstones are widely used in Chinese medicine. In this study, bile acids were used as chemical markers for the quality control of these drugs. Eighteen bile acids were simultaneously determined in six popular Chinese medicines by a fully validated LC/MS/MS method. The results revealed the difference of bile acids, both qualitatively and quantitatively, between bile-based Chinese medicines for the first time. The characteristic bile acids discovered in this study could be used to explicitly identify crude drugs even when they were component ingredient in complicated preparations. This study provides a general method for the quality control of bile-based Chinese medicines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.116.

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