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A tandem mass spectrometric study of bile acids: Interpretation of fragmentation pathways and differentiation of steroid isomers

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ABSTRACT

Bile acids are steroids with a pentanoic acid substituent at C-17. They are the terminal products of cholesterol excretion, and play critical physiological roles in human and animals. Bile acids are easy to detect but difficult to identify by using mass spectrometry due to their poly-ring structure and various hydroxylation patterns. In this study, fragmentation pathways of 18 free and conjugated bile acids were interpreted by using tandem mass spectrometry. The analyses were conducted on ion trap and triple quadrupole mass spectrometers. Upon collision-induced dissociation, the conjugated bile acids could cleave into glycine or taurine related fragments, together with the steroid skeleton. Fragmentations of free bile acids were further elucidated, especially by atmospheric pressure chemical ionization mass spectrometry in positive ion mode. Aside from universally observed neutral losses, eliminations occurred on bile acid carbon rings were proposed for the first time. Moreover, four isomeric 5β-cholanic acid hydroxyl derivatives (3α,6α-, 3α,7β-, 3α,7α-, and 3α,12α-) were differentiated using electrospray ionization in negative ion mode: 3α,7β-OH substituent inclined to eliminate H_2O and CH_2O_2 groups; 3α,6α-OH substituent preferred neutral loss of two H_2O molecules; 3α,12α-OH substituent apt to lose the carboxyl in the form of CO_2 molecule; and 3α,7α-OH substituent exhibited no further fragmentation after dehydration. This study provided specific interpretation for mass spectra of bile acids. The results could contribute to bile acid analyses, especially in clinical assays and metabonomic studies.

1. Introduction

Bile acids (BAs) are a group of steroids bearing a pentanoic acid side chain at C-17. They represent characteristic constituents of human and animal bile, and play critical roles in lipid absorption and metabolism [1,2]. They are signaling molecules which reflect internal secretion, especially during diseases and medications [3]. These characters made BAs important target analytes in physiological and pathological tests, as well as marker compounds in metabonomic studies. In addition, some BAs are used therapeutically in clinical practice [1,3]. Therefore, simple and sensitive methods to detect and identify BAs in complex body fluids are required.

Mass spectrometry (MS) is the most powerful technique to detect BAs due to its high sensitivity and abundant structural information [2,4,5]. Up to date, various MS ionization techniques including atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) and fast atom bombardment have been employed for the analysis of BAs, in combination with different mass analyzers like ion trap (IT), triple quadrupole (QqQ), time-of-flight (TOF), and Fourier transform ion cyclotron resonance [6–10]. A number of literatures have reported the fragmentation pathways of bile acids and their glutathione thioesters [11], 3-sulfates [12], coenzyme A esters [13], 24-glucosides [14], and synthesized derivatives [15]. MS behaviors of other free steroids were reported as well [10,16–23]. It was believed that minor difference in steroids structures, such as a double bond or a methyl group could result in distinctive fragmentation patterns [17]. However, details in the tandem mass spectrometry fragmentation of free bile acids, especially those commonly present in bio-fluids, remain unclear. As a result, accurate and fast identification of bile acids in biosamples remains to be a challenge.

Collision-induced dissociation (CID) provided abundant structural information, especially charge-remote fragmentation products [16,17]. In the present work, CID fragmentation pathways of 18 BAs were investigated, including six free BAs and their glycine and taurine conjugates. These are the most important bile acids

Abbreviations: APCI, atmospheric pressure chemical ionization; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CID, collision-induced dissociation; DCA, deoxycholic acid; ESI, electrospray ionization; HDCA, hyodeoxycholic acid; IT, ion trap; LCA, lithocholic acid; MS, mass spectrometry; QqQ, triple quadrupole; TOF, time-of-flight; UDCA, ursodeoxycholic acid.

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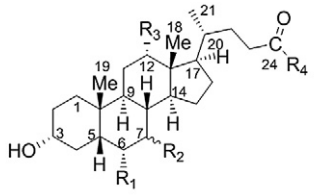
in mammal bile [24,25], and they possess the same 5β-cholanic acid backbone. Among them, cholic acid (CA) possesses three hydroxyl groups (3α,7α,12α-OH); chenodeoxycholic acid (CDCA, 3α,7α-OH), deoxycholic acid (DCA, 3α,12α-OH), hyodeoxycholic acid (HDCA, 3α,6α-OH) and ursodeoxycholic acid (UDCA, 3α,7β-OH) bear two hydroxyl groups in different positions; and lithocholic acid (LCA) carries only one hydroxyl group (3α-OH). Various MS techniques were employed, i.e. APCI and ESI ionizations together with QqQ, IT and TOF analyzers. With the combination of tandem mass spectrometry and high-resolution mass spectrometry, fragmentation pathways of BAs were studied in detail to allow their rapid and accurate identification. The results could be helpful to establish analytical methods in bile acid analyses, such as biological and clinical tests, as well as compound identification in metabonomic studies.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, methanol, formic acid (Mallinkrodt Baker, NJ, USA) and ammonium acetate (Sigma–Aldrich, MO, USA) were of HPLC grade. De-ionized water was prepared by a Milli-Q system (Millipore, MA, USA).

CA, CDCA, and DCA were purchased from the China National Institutes for Food and Drug Control (Beijing, China). LCA was purchased from Sigma–Aldrich (MO, USA). HDCA was from Tianqi Chemical Engineering (Anhui, China). UDCA was from Bio Basic Inc. (Ontario, Canada). Taurine and glycine conjugated bile acids were synthesized as previously reported by our group [26]. Their structures (Fig. 1) were identified by NMR spectroscopy and mass spectrometry [26]. Purities of all analyzed BAs were above 98%.



Compd.	R_1	R_2	R_3
Unconjugated BAs: R_4=OH			
CA	H	α-OH	OH
CDCA	H	α-OH	H
DCA	H	H	OH
HDCA	OH	H	H
LCA	H	H	H
UDCA	H	β-OH	H
Glycine-conjugated BAs: R_4=$NHCH_2CO_2H$			
GCA	H	α-OH	OH
GCDCA	H	α-OH	H
GDCA	H	H	OH
GHDCA	OH	H	H
GLCA	H	H	H
GUDCA	H	β-OH	H
Taurine-conjugated BAs: R_4=$NH(CH_2)_2SO_3H$			
TCA	H	α-OH	OH
TCDCA	H	α-OH	H
TDCA	H	H	OH
THDCA	OH	H	H
TLCA	H	H	H
TUDCA	H	β-OH	H

Fig. 1. Chemical structures of bile acids analyzed in this study.

2.2. Ion trap mass spectrometry

A Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, CA, USA) was operated in continuous infusion mode. The sheath and auxiliary gas was high purity nitrogen, and the collision gas was ultra-high purity helium. Parent mass range was set at *m*/*z* 100–1000, and the isolation width was 2.0 Th. Parameters for ESI negative mode were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 5 units; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, −18 V; tube lens offset voltage, −60 V, collision energy for CID, varied from 0% to 60%. Parameters for APCI positive mode were as follows: sheath gas, 40 units; auxiliary gas, 10 units; discharge current, 5 μA, capillary temperature, 175 °C; vaporizer temperature, 450 °C; capillary voltage, 7 V; tube lens offset voltage, 45 V; collision energy for CID, 30%. Pure BAs solutions (0.3 mg/mL in methanol) were injected into the instrument via a syringe pump at 5 μL/min combined on-line with a 50 μL/min flow of methanol. The instrument was controlled by Xcalibur 1.4™ software (Thermo Fisher Scientific, CA, USA).

To confirm the elemental composition of fragment ions with high-accurate mass, ion trap-time of flight mass spectrometry was employed. The system consisted of an LC-20AD pump, a SIL-20AC autosampler, a CTO-20A column oven, an ESI source, and an IT–TOF mass spectrometer (Shimadzu, Tokyo, Japan). An Atlantis T3 column (5 μm, ID 4.6 × 250 mm) equipped with an XTerra MS C_{18} guard column (5 μm, ID 3.9 × 20 mm) (Waters, MA, USA) was used. BAs were chromatographically separated following our previously reported method after minor modifications [26]. The mobile phase consisted of acetonitrile (A) and 4 mM ammonium acetate in water (pH = 7.43) (B). The gradient elution program was as follows: 0–13 min, 29–30% A; 13–15 min, 30–32% A; 15–35 min, 32% A; 35–45 min, 32–55% A; 45–55 min, 55–85% A (v/v). The flow rate was set at 1.0 mL/min, and the post-column splitting ratio to source was 2:1. The instrument parameters were set as follows: collision and cooling gas, high purity argon (Ar); nebulizing gas, high purity nitrogen (N_2, 1.5 L/min); CDL temperature, 200 °C; interface voltage, 4.5/−3.5 kV (positive/negative mode); detector voltage, 1.70 kV; endcap acceleration, −4.0/3.5 V (positive/negative mode); flight tube voltage, −7.0/+7.0 kV (positive/negative mode); CID energy, 50%; CID gas, 50%. MS and MS^n scan range, *m*/*z* 220–1000; precursor ion isolation, 3.0 Th. Instrument calibration was performed with a methanol solution of glycoursodeoxycholic acid ($C_{26}H_{43}NO_5$). The data were recorded and processed by the LC/MS solution V3.41 software, including a chemical formula predict program.

2.3. Triple quadrupole mass spectrometry

A Finnigan TSQ Quantum triple quadrupole mass spectrometer with ESI interface (Thermo Fisher Scientific, CA, USA) was operated in continuous infusion mode. High purity nitrogen was used as the sheath (40 units) and auxiliary (5 units) gas; high purity argon was used as the collision gas (1.0 mTorr). Instrument parameters were as follows: spray voltage, 4.0 kV; capillary temperature, 350 °C; capillary offset, −35 V; source-fragmentation voltage, 10 V. Parent mass range, *m*/*z* 200–800. The instrument was tuned and controlled by Xcalibur 2.0.7™ software (Thermo Fisher Scientific, CA, USA).

3. Results and discussion

3.1. Ionization conditions for BAs

MS conditions were optimized on an IT-MS instrument using a representative free bile acid DCA (0.3 mg/mL). Different ion