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Factors affecting separation and detection of bile acids by liquid chromatography coupled with mass spectrometry at negative mode

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Abstract

Bile acids (BAs) are cholesterol metabolites with important biological functions. They undergo extensive host-gut microbial co-metabolisms during the enterohepatic circulation, creating a vast structural diversity and resulting in great challenges to separate and detect them. Based on the bioanalytical reports in the past decade, this work developed three chromatographic gradient methods to separate a total of 48 BA standards on BEH C18 column and HSS T3 column and accordingly unraveled the factors affecting the separation and detection of them by liquid chromatography coupled with mass spectrometry (LC-MS). It was presented that both the acidity and ammonium levels in mobile phases reduced the electrospray ionization (ESI) of BAs as anions of $[M-H]^-$, especially for those unconjugated ones without 12-hydroxylation. It was also found that the retention of taurine-conjugates on BEH C18 column was sensitive to the strength of formic acid and ammonium in mobile phases. By using the volatile buffers with an equivalent ammonium level as mobile phases, we comprehensively demonstrated the effects of the elution pH value on the retention behaviors of BAs on both BEH C18 column and HSS T3 column. Based on the retention data acquired on C18 column, we presented the ionization constants (pK_a) of various BAs with the widest coverage beyond previous reports. When we made attempts to establish the structure-retention relationships (SRR) of BAs, the lack of discriminative structural descriptors for BA stereoisomers emerged as the bottleneck problem. The methods and results presented in this work are especially useful for the development a reliable, sensitive, high-throughput and robust LC-MS bioanalytical protocols for the quantitative metabolomic studies.

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Keywords

High Performance Liquid Chromatography; Mass Spectrometry; Bile acid; Electrospray ionization; Ionization constant; Structure-retention relationship

1. Introduction

Human Bile acids (BAs) are C₂₄ molecules comprised of a C₁₉ cyclopentanophenanthrene (steroid) nucleus and a carboxylate side-chain. They are cholesterol metabolites undergoing extensive enterohepatic circulation driven by a series of host-gut microbial metabolism and transport mechanisms [1–3]. During exchanges between the host and gut microbiota, BAs act on both of them and plays multiple biological roles [4–10]. The metabolomic profile of BAs therefore manifests a footprint of host-gut microbial interactions that are closely associated with health and diseases. However, the highly interactive disposition of BAs by the host and gut microbiota produces a vast structural diversity (Fig. 1 and Table S1) associated with (1) A/B ring fusion stereochemistry (trans/5 α -H or cis/5 β -H); (2) sites of hydroxylation at C₃, C₆, C₇ and/or C₁₂; (3) dehydrogenation and epimerization of the hydroxyl groups; and (4) conjugation of glycine or taurine at the C₂₄-carboxyl group and/or conjugation of glucuronide or sulfate at hydroxyl/ C₂₄-carboxyl groups [11]. It is challenging to separate and detect such a great deal of BAs in biological samples with such a small structural difference but disparate physicochemical properties.

Liquid chromatography coupled with mass spectrometry (LC-MS) is currently prevailing in BAs analysis [12]. In a typical LC-MS method, BAs are separated by reverse chromatography, ionized by the negative electrospray ionization (ESI⁻), and detected by multiple reaction monitoring (MRM). Owing to the occurrence of a lot of isomers and stereoisomers, it's difficult to differentiate them with current tandem mass spectrometric techniques. A pseudo-MRM method has to be employed for the unconjugated isomers, such as CDCA/HDCA, and most of unconjugated stereoisomers, such as LCA/isoLCA, CDCA/UDCA/ β UDCA, HDCA/ β HDCA/muroCA and HCA/ α MCA/ β MCA/ ω MCA. All these unconjugated BAs have no 12-hydroxyl group. They fragmented merely via dehydration and dehydrogenation and exhibit none discriminative fragments regardless of sites and epimerization of the hydroxyl groups on the skeleton [13]. We have characterized a distinctive fragmentation mechanism for the 12-hydroxylated unconjugated ones, such as DCA and CA. The 12-hydroxyl group induces the rotation of the carboxylate side chain and the proton transfer between 12-hydroxyl group and 24-carboxyl group. The subsequent dissociation routes enable the discrimination of 12-hydroxylated ones from the others with MRM method [13]. For the conjugated BAs, such as glycine-conjugates and taurine-conjugates, the dissociations of the steroid nucleus have been obscured by the signals derived from cleavage of the amide side chain [13]. As a result, isomers and stereoisomers of conjugated BAs may also not be discriminated by MRM method. In summary, the discrimination of the unknown BAs from the known ones relies heavily on the separation power and the robustness of chromatography. It is crucial to disclose the fundamental factors affecting the separation and detection of them and accordingly establish structure-retention relationships (SRR).

Table S2 summarized the published chromatographic methods for BAs separation in the past decade. At the side of stationary phases, the reverse C18 columns were utilized in the majority of reports. The High Strength Silica (HSS) T3 columns were employed in several reports and only two reports utilized a reverse C8 column. The situation became much more complicated at the side of mobile phases. Some reports only used formic acid (HCOOH) in aqueous phase and/or organic phase. A few others used ammonium acetate ($\text{CH}_3\text{COONH}_4$) but the pH value of mobile phases varied from 4.0 to 9.0. It is not completely clear how the additives and pH value of mobile phases affect the separation and detection of BAs on a specific stationary phase. Therefore, the first aim of this study was to disclose the factors that have potential impacts on the separation and detection of BAs by LC-MS. By using the collected data, the second aim of this work was to highlight the challenges for understanding the fundamental relationship between the structure and physicochemical property of BAs, which will play pivotal role in the characterization and identification of the unknown BAs detected in human and animals.

2. Material and methods

2.1. Chemicals and reagents

Forty eight BA reference standards were purchased from Steraloids (Newport, RI, USA), TRC (Toronto, Canada), or Sigma-Aldrich (St. Louis, MO, USA). The authentic standard of 3β -ursocholic acid (β UCA) was kindly gifted from Prof. Dr. Takashi Iida (Nihon University). The name, chromatographic peak label, CAS, m/z of $[\text{M}-\text{H}]^-$ and retention data of them were summarized in Table 1. The LC-MS grade methanol (MeOH), acetonitrile (ACN), isopropanol alcohol (IPA), formic acid (HCOOH), ammonium formate (HCOONH_4), ammonium acetate ($\text{CH}_3\text{COONH}_4$), ammonium bicarbonate (NH_4HCO_3), ammonium hydroxide ($\text{NH}_3\cdot\text{H}_2\text{O}$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was obtained by using a Milli-Q system (Millipore, Bedford, USA). The 5 mM stock solutions of each BA standard were individually prepared in methanol. The individual standard samples and the mixed standard samples were prepared at 5 μM by diluting the stock solutions with water-acetonitrile (50:50, v/v).

2.2. Preparation of volatile buffers

According to our previous protocols [14], the volatile buffers used as mobile phases were prepared with SevenExcellence S400 pH/mV Meters (Mettler-Toledo, Columbus, OH, USA) under magnetic stirring. The buffers at pH 2.5, 3.0, 3.5 and 4.0 were prepared using 2 mM HCOONH_4 and formic acid solutions, the buffers at pH 4.5, 5.0 and 5.5 were prepared using 2 mM $\text{CH}_3\text{COONH}_4$ and acetic acid solutions, the buffers at pH 6.0, 6.5, 7.0 and 7.5 were prepared using 2 mM NH_4HCO_3 and ammonium solutions, the buffer at pH 8.5 was prepared using 2 mM $\text{CH}_3\text{COONH}_4$ and ammonium solutions. The buffers with different ammonium strength were prepared by similar methods. All buffers were freshly prepared previous to be used as mobile phases.

2.3. Chromatography

The chromatographic separation was performed on a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA). The Ethylene Bridged Hybrid (BEH) C18 column and the

HSS T3 column were comparatively used in this work. Three chromatographic methods (Gradient I, II and III) derived from or optimized from our previous studies were employed in this work [15,13]. The gradients of the three methods were summarized and illustrated on Fig. S1. The individual standard sample was injected to acquire basic retention behavior of each analyte under each method. The mixed standard samples were injected during method optimization and switching of mobile phases. The system was equilibrated for at least 30 min after switching mobile phases and at least five injections of the mixed standard sample were repeatedly analysed thereafter. The first two injections were used to confirm equilibrium of system and the other three injections were used to record the retention behaviors. The injection volume was 5 μ L.

The Gradient-I with a run time of 20 min utilized ACQUITY BEH C18 column (1.7 μ m, 100 mm $\times$ 2.1 mm) (Waters, Milford, MA, USA) maintained at 45 $^{\circ}$ C. The mobile phases consisted of 0.1% HCOOH in water (mobile phase A) and 0.1% HCOOH in ACN-MeOH (95:5, v/v, mobile phase B). The flow rate was 0.45 mL/min. The gradient program was 0.0–0.5 min (5% B), 0.5–1.0 min (5–20% B), 1.0–2.0 min (20–25% B), 2.0–5.5 min (25% B), 5.5–6.0 min (25–30% B), 6.0–8.0 min (30% B), 8.0–9.0 min (30–35% B), 9.0–17.0 min (35–65% B), 17.0–18.0 min (65–100% B), 18.0–19.0 min (100% B) and 19.0–20.0 (5% B).

The Gradient-II with a run time of 14 min employed the same column of Gradient-I maintained at 45 $^{\circ}$ C. The mobile phases consisted of 0.01% HCOOH in water (mobile phase A) and ACN (mobile phase B). The flow rate was 0.45 mL/min. The gradient program was 0.0–0.5 min (5% B), 0.5–1.0 min (5–35% B), 1.0–4.0 min (35–30% B), 4.0–5.0 min (30% B), 5.0–6.0 min (30–40% B), 6.0–8.0 min (40–45% B), 8.0–11.5 min (45–70% B), 11.5–12.0 min (70–100% B), 12.0–13.0 min (100% B), 13.0–13.1 min (100–5% B) and 13.1–14.0 (5% B).

The Gradient-III with a run time of 25 min used an ACQUITY HSS T3 column (1.8 μ m, 100 mm $\times$ 2.1 mm) (Waters, Milford, MA, USA) maintained at 35 $^{\circ}$ C. The mobile phases consisted of 2mM HCOONH₄ in water (pH 3.0 adjusted by HCOOH, mobile phase A) and ACN-MeOH-IPA (8:1:1, v/v/v, mobile phase B). The flow rate was 0.40 mL/min. The gradient program was 0.0–0.3 min (5% B), 0.3–1.0 min (5–10% B), 1.0–4.0 min (10–35% B), 4.0–6.0 min (35% B), 6.0–12.0 min (35–40% B), 12.0–18.0 min (40–45% B), 18.0–20.0 min (45–60% B), 20.0–22.0 min (60–80% B), 22.0–23.5 min (80–100% B), 23.5–24.4 min (100% B), 24.4–24.5 min (100–5% B) and 24.5–25.5 (5% B).

2.4. Mass Spectrometry

The tandem mass spectrometric analysis was performed on a Xevo G2S Q-TOFMS via an ESI interface (Waters, Milford, MA, USA) operated at negative mode. The capillary voltage was 3.0 kV. The source and desolvation temperature was 150 and 550 $^{\circ}$ C, respectively. Nitrogen and argon were used as cone and collision gases, respectively. The cone gas flow and desolvation gas flow was respectively set at 50 and 950 L/h. The detection of BAs was conducted by MS/MS scans (m/z 50–600, centroid mode, scan time 0.036 s, interscan time 0.014 s) for the deprotonated quasi-molecular ions of analytes, [M-H]⁻. The collision energy (CE) was set at 15 V for unconjugated-BAs and glycine-conjugates, and 35 V for taurine-

conjugates, sulfates and glucuronides. Leucine enkephalin was infused via the reference probe as lockspray at both negative and positive mode to ensure m/z accuracy.

$$k' = (t_R - t_0)/t_0 \quad (1)$$

$$k' = \frac{k'_{HA} * 10^{-pH} + k'_{A^-} * 10^{-pKa}}{10^{-pH} + 10^{-pKa}} \quad (2)$$

2.5. Data processing

The raw data was processed by MassLynx (V4.1, Waters, Milford, MA, USA). The ion chromatogram of BAs was acquired by extracting m/z data at the pseudo-MRM transition ($[M-H]^- > [M-H]^-$) according to Table 1. Peak integration was carried out under automatic noise measurement and smoothing (mean method, twice at the window size of ± 1 scan). The reported retention time (t_R), peak height and peak area were the mean data from triplicate analysis. Capability factor (k') was calculated according to (Equation 1), where t_R was the retention time of a BA under a specific gradient and t_0 was the dead time of the system. Since most of the BAs are monobasic acids, non-linear curve fitting according to (Equation 2) [16] was carried out by OriginPro 9.0 (OriginLab, Northampton, MA, USA) for the dataset of k' and pH value of the aqueous phases to simulate the ionization constants (pKa) data of them.

3. Results and Discussions

3.1. Chromatographic developments

Three basic chromatographic methods were employed into this work. The 20-min Gradient-I on an ACQUITY BEH C18 column (1.7 μm , 100 mm $\times$ 2.1 mm) has been used in our previous works for disclosing the negative fragmentation behaviors of BAs [13]. The 14-min Gradient-II with an extraordinary gradient program was particularly optimized to separate the unconjugated BAs on the same column. Fig. 2 illustrated the key intermediate gradient programs and the corresponding retention time of unconjugated BAs during gradient optimization. It was highlighted the challenges to separate some isomers of unconjugated BAs, especially for the trihydroxyl ones, such as ω MCA and β CA that were co-eluted under the Gradient-I with a longer run-time. The 25-min Gradient-III was developed on an ACQUITY HSS T3 column (1.8 μm , 100 mm $\times$ 2.1 mm) for the better retention of the polar BA species. All the three methods were optimized with the principle goal of achieving accepted resolution with the least run time. The retention time of various BAs on the three chromatographic methods and the corresponding percentage of organic phase at their retention time were listed in Table 1.

3.2. Formic acid in mobile phases

Fig. 3(a) comparatively illustrated the chromatographic retention data of 32 BAs when formic acid was modified in mobile phases of the Gradient-I. The chromatographic behavior was unacceptable when formic acid was deprived from both phases. When formic acid was deprived from either phase, the retention time of unconjugated BAs and glycine-conjugates remained unchanged, but those of taurine-conjugates increased. Similar phenomenon was observed when 0.1% or 0.01% formic acid was added only in the aqueous phase. The retention time of taurine-conjugates significantly increased with the decreased acidity while those of glycine-conjugates and unconjugates remained stable.

The different effects of acidity on the retention of the three panels of BAs were associated with their pK_a value. The unconjugated BAs and taurine-conjugates usually have a pK_a value of 5~6 and 4~5^[4], respectively. The majority of them remained unionized in 0.01~0.1% HCOOH (pH 2.7~3.3), which explained the stabilities of their retention time under these conditions. In contrast, most of taurine-conjugates was dissociated in the mobile phase because their pK_a value is less than 2.0^[4]. Therefore, in association with acidity of mobile phases, there are some other mechanisms that affect the retention behaviors of the ionized taurine-conjugates on C18 column. It was demonstrated that the octanol/water partition coefficients of ionized BAs increased with Na⁺ concentration [17] and the capacity factors of the fully ionized BAs increased with increasing ionic strength[18]. As trace of Na⁺ occurs in the LC-MS system, it was proposed that the enhanced retention of taurine-conjugates be ascribed to the formation of Na⁺-complexes of BA anions under low acidity.

Fig. 3(b) comparatively illustrated the peak height data of 32 BAs when formic acid was modified in mobile phases of the Gradient-I. The ESI efficacies of BAs were significantly inhibited by the acidity of mobile phases. The removal of formic acid in either phase enhanced the responses of all BAs. When formic acid was added only in the aqueous phase, a low acidity (0.01%) significantly increased the responses of all BAs compared with a high acidity (0.1%). Those BAs with the most enhanced intensities were the unconjugated BAs without a 12-hydroxylation, such as LCA, CDCA, HDCA, UDCA, β UDCA, HCA, muroCA, ω MCA, β MCA and α MCA. In association with our recent negative-ion fragmentation studies[19], the phenomenon might be associated with the lack of 12-hydroxyl group of these BAs. Without an intra-molecular hydrogen bond between 12-hydroxyl group and 24-carboxylate, the negative charge of these BAs was unstable and more sensitive to ionization suppression by additives in the mobile phases. The above observations indicated that an appropriate acidification of mobile phases facilitates the separation of BAs on the reverse column but a high acidity would significantly inhibit the negative ionization of them, especially the unconjugated BAs without 12-hydroxylation.

3.3. Ammonium in mobile phase

Ammonium salts are widely-used volatile additives allowed for LC-MS analysis. The effects of ammonium on ESI efficacies of BAs have been preliminarily demonstrated while the factors derived from the pH value of infusion vehicles were not analyzed [20]. Therefore, we firstly investigated the effects of ammonium on the separation and detection of unconjugated BAs based on the Gradient-II by switching the aqueous phase as 0.008%, 0.01% and

0.012% HCOOH and pH 3.5, 4.0 and 4.5 HCOONH₄ buffers (2 mM), respectively. The pH value of 0.008% HCOOH had an approximately equivalent pH value to the pH 3.5 HCOONH₄ buffer. Fig. 5 showed the typical ion chromatograms of unconjugated BAs eluted under the tested conditions. The retention time of unconjugated ones did not vary while switching the aqueous phases. However, as indicated by the peak area data, the ionization of them as [M-H]⁻ was significantly inhibited by ammonium in the mobile phases (Fig. 4).

The effects of ammonium on the separation and detection of conjugated BAs were similarly investigated based on the Gradient-II. As shown in Fig. S3, the ESI ionizations of conjugated BAs as [M-H]⁻ were also inhibited by the ammonium in the mobile phases. In addition, the retention time of them eluted by pH 3.5 HCOONH₄ buffer was much shorter than that eluted by 0.01% HCOOH despite that the two aqueous phases had an equivalent pH value. The retention variations of taurine-conjugates were more significant than those of glycine conjugates. We therefore additionally investigated the effects of ammonium concentrations on the separation of conjugated BAs based on the Gradient-I. Fig. S4 showed the ion chromatograms acquired by using the pH 3.5 buffers prepared from 0.2 mM, 2.0 mM and 20 mM ammonium formate, respectively. The results were consistent with previous finding that ammonium attenuate the retention of conjugated BAs. The phenomenon might also be associated with the inhibition of Na⁺-complexes of them by ammonium. The above observations indicated that the ESI ionizations of all BAs as [M-H]⁻ and the chromatographic retention of conjugated BAs are to be inhibited by the ammonium in mobile phases.

3.4. pH of mobile phase

The above results clearly presented that both the acidities and the ammonium in mobile phases have a significant impact on the separation and detection of BAs by LC-MS. Therefore, the affecting factor derived from ammonium has to be well controlled while investigating the effects of pH value of aqueous phases on chromatographic retention of BAs. Therefore, the aqueous phases at various pH were prepared in parallel based on 2 mM volatile buffers. The tests for unconjugated BAs and conjugated BAs were carried out based on the Gradient-II and the Gradient-I, respectively. As illustrated in Fig. 5 and Fig. 6, the chromatographic retention of both unconjugated and conjugated BAs decreased with increasing pH of mobile phase. As a result, the resolution of some unconjugated isomers greatly decreased, especially for the trihydroxyl and dihydroxyl unconjugated BAs, such as ωMCA and βCA, muroCA and βUDCA, UDCA and HDCA. As shown in Fig. 7, the retention variations of three panels of BAs with the pH value of mobile phases were different. The retentions of unconjugated BAs varied the most within pH 4.0–6.0, the retentions of glycine-conjugates varied the most within pH 3.5–6.5, and the retentions of taurine -conjugates remained limited variation within pH 2.5– 6.5.

3.5. Stationary phase

BEH C18 column and HSS T3 column were two stationary phases the most frequently used for BAs analysis in the past decade. In order to compare the retention behaviors of BAs on the two columns, we additionally investigated the effects of pH value of aqueous phases on

chromatographic retention of BAs under Gradient-III using a HSS T3 column. Several differences in the retention behaviors of BAs were observed on the two columns (Fig. 7). Firstly, the retention variations of polar BAs with elution pH were more significant on the HSS T3 column than those on the BEH C18 column, especially for taurine-conjugated ones, the most polar panel of BAs. Secondly, CDCA-3G and CDCA-24G, another two polar BA metabolites, were not separated on BEH C18 column but separated on HSS T3 column in the acidic mobile phases with pH value less than 4.0. Finally, CA was more retained than ACA on the BEH C18 column, while ACA was more retained than CA on the HSS T3 column.

These observations indicated that the separation mechanisms on HSS T3 column involve more mechanism beyond the classic partition theories and possibly associated with the specific pore structure. The ligand density, carbon load and surface area are $3.1 \mu\text{mol}/\text{m}^3$ and $1.6 \mu\text{mol}/\text{m}^3$, 18% and 11%, and $185 \text{ m}^2/\text{g}$ and $230 \text{ m}^2/\text{g}$ for BEH C18 particles and HSS T3 particles, respectively. Compared to the other silica-based materials, the analytes may more readily access the pore structure of HSS T3 particles with the lower carbon load, providing more retention of polar molecules. The proposed mechanism explained to some extent the different relative retention of CA/ACA and CDCA-3G/CDCA-24G on HSS T3 column compared to BEH C18 column.

3.6. Ionization constants of BAs

According to the relationships between capacity factors and the pH of the mobile phase [21,16], we may simultaneously determine the pK_a of various BAs. This method requires only a small quantity of compounds and is particularly useful for simulating the pK_a data necessary for the optimization of chromatographic separations. Similar works including limited species of BAs were published in the last century [22,18,17]. Because the separation mechanisms on HSS T3 column are beyond the classic partition theories, the data acquired on BEH C18 column other than HSS T3 column was suitable for this purpose according to the fundamental of (Equation 2) [16]. Fig. 8 illustrated the none-linear curve fitting for the majority of BAs except for the taurine-conjugates, CDCA-3G and LCA-3S. Simulation for taurine-conjugates was not obtained because it's difficult to prepare the volatile buffers with $\text{pH} < 2.0$ and the pK_a of taurine-conjugates was less than 2.0. Simulation for CDCA-3G and LCA-3S was not performed because they are not monobasic acids. The simulated pK_a value was listed in Table 2.

The pK_a data obtained in this work was a little different from data acquired from the potentiometric methods [4]. The major reason for this difference was the presence of organic solvents in mobile phases. It has been shown that the effect of organic solvents, such as methanol [23,24], acetonitrile [25] and tetrahydrofuran [26], on dissociation constants manifests markedly only at relatively high levels above about 80%. An increase of the pK_a values was found in the range of 1.5–2.0 pK units with increasing concentration of ACN up to 80% [27], and similar values was found for IPA [28]. In this regard, one should bear in mind that the more the pK_a data listed in Table 2 was deviated from those obtained by the potentiometric method when the analyte was more retained on the C18 column according to the data present in Table 1.

3.7. Challenge for structure-retention relationship studies

The SRR is of importance not only for retention prediction and analyte identification but also for evaluation of informative descriptors related to the physicochemical and biological properties [29,30]. However, the SRR studies of BAs were still limited in some major species without consideration of stereochemistry [17,31,32]. Our previous work has compared the retention time of 15 pairs of BA stereoisomers under the 20-min Gradient-I on a BEH C18 column [13]. It was shown that BAs containing a β -hydroxyl group generally had shorter retention time than the corresponding isomer with an α -hydroxyl group except for α MCA and β MCA and their taurine conjugates. LogP is one of the most important structural descriptors to develop SRRs for the reverse liquid chromatography [29,30]. We collected the predicted LogP data from various sources (Table S1), including XLogP3 (PubChem), ALogPS and ChemAxon-LogP (HMDB), ACD/LogDpH5.5 (Chemspider), and LogP and CLogP (ChemBioDraw14). Unfortunately all the algorithms are unable to differentiate the stereoisomers. The same situations appeared for the other structural descriptors, such as polar surface area, as well as in a recent quantitative SRR study including only one pair of stereoisomers (CDCA and UDCA) [32].

Owing to the lacking of discriminative structural descriptors, there are currently no effective methods to develop the quantitative SRRs of various BAs including the stereoisomers. In a simple manner, the correlation between the LogP and retention time (t_R) was evaluated for those BAs with the cis A/B ring juncture (5β) and α -hydroxyl groups. The results for a total of 35 analytes were shown in Fig. 9. It was demonstrated that XLogP3 and ClogP exhibited the best linear correlation ($r > 0.85$) for this them. Although the SRR studies of BA stereoisomers are still challenging, the visual analysis of the retention data of BA stereoisomers and their 3D structures proposed that the mechanism be associated with structural variations that potentially disturb the unique amphipathic conformation of BAs. Anyway, the SRR studies of BAs are in urgent need of the structural descriptors algorithm with capability of differentiating stereoisomers.

4. Conclusion

In summary, this work developed three chromatographic gradients to disclose the miscellaneous factors affecting the separation and detection of BAs by liquid chromatography coupled with mass spectrometry. The effects of pH value and ammonium levels of mobile phases on the retention behaviors and the negative electrospray ionization of various kinds of BAs including unconjugated ones, glycine-conjugates, taurine-conjugates, sulfates and glucuronides were comprehensively demonstrated. Based on the retention data acquired on C18 column, the ionization constants (pK_a) of BAs with the widest coverage beyond previous reports have been presented. Our attempts to establish the SRR of BAs has highlighted that the SRR studies of BAs are in urgent need of the structural descriptors algorithm with capability of differentiating stereoisomers. The methods and results in this work are especially useful for the developments of reliable, sensitive, high-throughput and robust LC-MS bioanalytical protocols for the quantitative metabolomic studies of BAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Chemical structure of C24 bile acids. The mostly known structural diversity appears at (1) A/B ring fusion stereochemistry (trans/5 α -H or cis/5 β -H); (2) sites of hydroxylation at C3, C6, C7 and/or C12; (3) dehydrogenation and epimerization of the hydroxyl groups; and (4) conjugation of glycine or taurine at the C24-carboxyl group and/or conjugation of glucuronide or sulfate at hydroxyl/ C24-carboxyl groups.

Fig. 2.

Retention time of unconjugated BAs eluted by the key intermediate gradient programs during optimization of the Gradient-II on an ACQUITY BEH C18 column. Mobile phase A: 0.01% formic acid (v/v) in water; Mobile phase B: acetonitrile; Blue square: monohydroxyl-BAs (isoLCA/alloLCA/LCA, blue square); Green diamond: dihydroxyl-BAs (muroCA/βUDCA/UDCA/HDCA/CDCA/DCA/isoDCA); Red circle: trihydroxyl-BAs (βUCA/UCA/ωMCA/βCA/αMCA/βMCA/HCA/ACA/CA).

Fig. 3.

Effects of formic acid in mobile phases on the chromatographic retention (a) and negative ionization (b) of BAs. All tests were performed by using the Gradient-I. The ionization efficacy was indicated by peak heights. The peak height data of those BAs detected in the same channel but not fully separated was not available, such as the data of ω MCA/ β CA and some data of TUDCA/THDCA.

Fig. 4.

Effects of ammonium in mobile phases on the negative ionization of unconjugated BAs. All tests were performed under the Gradient-II by switching the aqueous phases as 0.008% formic acid, 0.01% formic acid and 0.012% formic acid and the ammonium formate buffers (2 mM) at pH 3.5, 4.0 and 4.5. The ionization efficacy was indicated by peak areas.

Fig. 5.

Ion chromatograms of unconjugated BAs on an ACQUITY BEH C18 column eluted by the Gradient-II with various buffers (from bottom to top: 0.01% formic acid, 2 mM ammonium formate at pH 3.5, 2 mM ammonium acetate at pH 4.5, 2 mM ammonium acetate at pH 5.5, 2 mM ammonium bicarbonate at pH 6.5 and 2 mM ammonium bicarbonate at pH 7.5) and acetonitrile as the aqueous phase and the organic phase respectively.

Fig. 6.

Ion chromatograms of conjugated BAs on an ACQUITY BEH C18 column eluted by the Gradient-I with various buffers (from bottom to top: 0.01% formic acid, 2 mM ammonium formate at pH 3.5, 2 mM ammonium acetate at pH 4.5, 2 mM ammonium acetate at pH 5.5 and 2 mM ammonium bicarbonate at pH 6.5) and acetonitrile as the aqueous phase and the organic phase respectively.

Fig. 7. Retention variations with the pH value of mobile phases for the unconjugated BAs (A, C) and the conjugated BAs (B, D) on BEH C18 column (A, B) and HSS T3 column (C, D).

Fig. 8.

Plot of capacity factors against the pH of the mobile phase on ACQUITY BEH C18 column for the unconjugated BAs (A), the glycine-conjugated BAs (B), taurine-conjugated BAs (C) and the other unconjugated and conjugated BAs (D). The data of conjugated BAs and unconjugated BAs was collected under the Gradient-I and Gradient-II, respectively.

Fig. 9.
Correlation between the predicted LogP and the retention time acquired by the Gradient-I for a total of 35 BAs with the cis A/B ring juncture (5 β) and without β -hydroxyl groups.

Table 1.

The peak No., name, abbreviated name, CAS, m/z of [M-H]⁻, retention time and the corresponding percentage of organic phase at its eluted time of bile acids included in this study.

No.	Name	Abbr.	CAS	[M-H] ⁻	Retention time (min) / percentage of organic phase (%)		
					Gradient-I ^a	Gradient-II ^b	Gradient-III ^c
1	lithocholic acid	LCA	434-13-9	375.3	17.17 / 71.0	11.75 / 85.0	23.16 / 95.5
2	isolithocholic acid	isoLCA	1534-35-6	375.3	15.95 / 61.1	10.91 / 67.2	22.49 / 86.5
3	allolithocholic acid	alloLCA	2276-93-9	375.3	15.65 / 59.9	11.11 / 65.8	22.60 / 88.0
4	isodeoxycholic acid	isoDCA	566-17-6	391.3	16.23 / 62.1	11.16 / 67.6	22.69 / 89.2
5	deoxycholic acid	DCA	83-44-3	391.3	14.55 / 55.8	09.93 / 58.8	21.79 / 77.9
6	chenodeoxycholic acid	CDCA	474-25-9	391.3	14.23 / 54.6	09.67 / 56.9	21.53 / 75.3
7	hyodeoxycholic acid	HDCA	83-49-8	391.3	12.17 / 46.9	07.61 / 44.0	18.13 / 46.0
8	ursodeoxycholic acid	UDCA	128-13-2	391.3	11.97 / 46.1	07.46 / 43.7	16.88 / 44.1
9	β-ursodeoxycholic Acid	βUDCA	78919-26-3	391.3	11.38 / 43.9	06.91 / 42.3	15.28 / 42.7
10	murocholic acid	muroCA	658-49-5	391.3	11.23 / 43.4	06.78 / 42.0	14.71 / 42.3
11	cholic acid	CA	81-25-4	407.3	11.93 / 46.0	07.31 / 43.3	17.45 / 44.5
12	allocholic acid	ACA	2464-18-8	407.3	11.82 / 45.6	07.17 / 42.9	17.65 / 44.7
13	hyocholic acid	HCA	547-75-1	407.3	11.23 / 43.4	06.67 / 41.7	15.13 / 42.6
14	β-muricholic acid	βMCA	2393-59-1	407.3	10.38 / 40.2	05.36 / 33.6	12.55 / 40.5
15	α-muricholic acid	αMCA	2393-58-0	407.3	10.09 / 39.1	04.55 / 30.0	11.89 / 39.9
16	3β-Cholicacid	βCA	3338-16-7	407.3	09.87 / 38.3	04.13 / 30.0	11.58 / 39.7
17	ω-muricholic acid	ωMCA	6830-03-1	407.3	09.82 / 38.1	03.98 / 30.0	11.26 / 39.4
18	ursocholic acid	UCA	2955-27-3	407.3	08.25 / 31.3	02.95 / 31.8	08.85 / 37.4
19	3β-ursocholicacid	βUCA	10322-18-6	407.3	05.95 / 29.5	02.22 / 33.0	06.46 / 35.4
20	glycolithocholic acid	GLCA	474-74-8	432.3	14.82 / 56.8	11.22 / 68.0	21.76 / 77.6
21	taurolithocholic acid	TLCA	516-90-5	482.3	14.23 / 54.6	10.22 / 60.9	22.70 / 89.3
22	glycodeoxycholic acid	GDCA	16409-34-0	448.3	12.63 / 48.6	08.11 / 45.8	19.24 / 54.3
23	glycochenodeoxycholic acid	GCDCA	16564-43-5	448.3	12.24 / 47.2	07.69 / 44.2	17.88 / 44.9
24	glycohyodeoxycholic acid	GHDCA	13042-33-6	448.3	10.07 / 39.0	04.35 / 30.0	11.38 / 39.5
25	glycoursodeoxycholic acid	GUDCA	64480-66-6	448.3	09.85 / 38.2	04.24 / 30.0	10.66 / 38.9
26	taurodeoxycholic acid	TDCA	207737-97-1	498.3	11.93 / 46.0	08.88 / 51.3	15.91 / 43.3
27	taurochenodeoxycholic acid	TCDCA	6009-98-9	498.3	11.45 / 44.2	08.21 / 46.5	14.48 / 42.1
28	taurohyodeoxycholic acid	THDCA	110026-03-4	498.3	08.65 / 33.3	04.74 / 30.0	08.85 / 37.4
29	taoursodeoxycholic acid	TUDCA	14605-22-2	498.3	08.46 / 32.3	04.47 / 30.0	08.48 / 37.1
30	glycocholic acid	GCA	475-31-0	464.3	10.17 / 39.4	03.04 / 31.6	11.71 / 39.8
31	glycohyocholic acid	GHCA	32747-08-3	464.3	08.97 / 34.9	04.52 / 30.0	09.43 / 37.9
32	taurocholic acid	TCA	81-24-3	514.3	09.21 / 35.8	05.32 / 33.2	09.56 / 38.0
33	taurohyocholic acid	THCA	117997-17-8	514.3	07.27 / 30.0	03.33 / 31.1	07.45 / 36.2
34	tauro β-muricholic acid	TβMCA	25696-60-0	514.3	05.60 / 26.0	02.73 / 32.1	05.99 / 35.0
35	tauro α-muricholic acid	TαMCA	25613-05-2	514.3	05.38 / 25.0	02.64 / 32.3	05.87 / 35.0
36	tauro ω-muricholic acid	TωMCA	NA	514.3	05.02 / 25.0	02.55 / 32.4	05.68 / 35.0

No.	Name	Abbr.	CAS	[M-H] ⁻	Retention time (min) / percentage of organic phase (%)		
					Gradient-I ^a	Gradient-II ^b	Gradient-III ^c
37	7-ketolithocholic acid	7-ketoLCA	4651-67-6	389.3	12.84 / 49.4	08.39 / 47.8	
38	6-ketolithocholic acid	6-ketoLCA	2393-61-5	389.3	12.35 / 47.6	07.86 / 44.7	
39	12-ketolithocholic acid	12-ketoLCA	5130-29-0	389.3	13.10 / 50.4	08.68 / 49.9	
40	3-dehydrocholic acid	3-DHCA	2304-89-4	405.3	11.51 / 44.4	06.97 / 42.4	
41	7-dehydrocholic acid	7-DHCA	911-40-0	405.3	10.30 / 39.9	05.16 / 31.6	
42	12-dehydrocholic acid	12-DHCA	2458-08-4	405.3	10.79 / 41.7	06.33 / 40.8	
43	apocholic acid	apoCA	641-81-6	389.3	13.33 / 51.2	08.85 / 51.1	
44	23-nordeoxycholic acid	NorDCA	53608-86-9	377.3	13.18 / 50.7	08.70 / 50.0	20.30 / 63.0
45	norcholic acid	NorCA	60696-62-0	393.3	10.34 / 40.0	04.93 / 30.0	12.24 / 40.2
46	Chenodeoxycholic acid 24-glucuronide	CDCA-24G	208038-27-1	567.3	12.12 / 46.7		17.70 / 44.8
47	Chenodeoxycholic acid 3-glucuronide	CDCA-3G	58814-71-4	567.3	12.10 / 46.6		16.64 / 43.9
48	Lithocholic acid 3-sulfate	LCA-3S	34669-57-3	456.6	15.10 / 57.9		21.62 / 76.2

^a: Acquired on ACQUITY BEH C18 column (1.7 µm, 100 mm × 2.1 mm) by Gradient-I (A: 0.1% formic acid in water, B: 0.1% formic acid in methanol: acetonitrile (10:90));

^b: Acquired on ACQUITY BEH C18 column (1.7 µm, 100 mm × 2.1 mm) by Gradient-II (A: 0.01% formic acid in water, B: acetonitrile);

^c: Acquired on ACQUITY HSS T3 column (1.8 µm, 100 mm × 2.1 mm) by Gradient-III (A: pH 3.0 ammonium formate buffer (2mM), B: methanol:isopropyl alcohol:acetonitrile(10:10:80))

Table 2.

Ionization constants of BAs determined by chromatographic methods.

Species	Ionization constant (mean $\pm$ standard error)			
	Unconjugate	Glycine-conjugate	Taurine-conjugate	24-glucuronide conjugate
LCA	5.81 $\pm$ 0.03	4.33 $\pm$ 0.01	<2.5	N/A
isoLCA	5.80 $\pm$ 0.04	N/A	N/A	N/A
alloLCA	5.83 $\pm$ 0.04	N/A	N/A	N/A
muroCA	5.36 $\pm$ 0.06	N/A	N/A	N/A
bUDCA	5.38 $\pm$ 0.06	N/A	N/A	N/A
UDCA	5.51 $\pm$ 0.06	4.24 $\pm$ 0.01	<2.5	N/A
HDCA	5.57 $\pm$ 0.06	4.32 $\pm$ 0.01	<2.5	N/A
CDCA	5.83 $\pm$ 0.03	4.29 $\pm$ 0.01	<2.5	3.68 $\pm$ 0.03
DCA	5.80 $\pm$ 0.02	4.27 $\pm$ 0.02	<2.5	N/A
isoDCA	5.65 $\pm$ 0.04	N/A	N/A	N/A
bUCA	5.26 $\pm$ 0.03	N/A	N/A	N/A
UCA	5.20 $\pm$ 0.03	N/A	N/A	N/A
wMCA	5.17 $\pm$ 0.03	N/A	<2.5	N/A
bCA	5.12 $\pm$ 0.03	N/A	N/A	N/A
aMCA	5.15 $\pm$ 0.03	N/A	<2.5	N/A
bMCA	5.14 $\pm$ 0.03	N/A	<2.5	N/A
HCA	5.31 $\pm$ 0.06	4.24 $\pm$ 0.04	<2.5	N/A
ACA	5.39 $\pm$ 0.06	N/A	N/A	N/A
CA	5.42 $\pm$ 0.06	4.35 $\pm$ 0.01	<2.5	N/A
apoCA	5.63 $\pm$ 0.06	N/A	N/A	N/A
NorDCA	5.61 $\pm$ 0.06	N/A	N/A	N/A
NorCA	5.06 $\pm$ 0.03	N/A	N/A	N/A
6-ketoLCA	5.58 $\pm$ 0.06	N/A	N/A	N/A
7-ketoLCA	5.64 $\pm$ 0.07	N/A	N/A	N/A
12-ketoLCA	5.59 $\pm$ 0.08	N/A	N/A	N/A
7DHCA	5.09 $\pm$ 0.03	N/A	N/A	N/A
3DHCA	5.34 $\pm$ 0.06	N/A	N/A	N/A