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Short communication

Ionization of unconjugated, glycine- and taurine-conjugated bile acids by electrospray ionization mass spectrometry

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Abstract

We investigated the effect of organic anions as spray liquid additives on the ionization efficiency of unconjugated, glycine-conjugated and taurine-conjugated bile acids under electrospray ionization conditions. Addition of organic acids influenced the ionization efficiency of whole bile acids. Use of a stronger acid reduced the peak intensity of unconjugated and glycine-conjugated bile acids, while the use of TFA, the strongest acid tested, improved the intensity of taurine conjugates. The hydroxyl group at the C-12α position of cholic acid and deoxycholic acid easily underwent intra-molecular hydrogen bonding with the side chain carboxyl group, accelerating the ionization efficiency. This intra-molecular hydrogen bond may also affect the formation of product ions in low energy-CID. The addition of ammonium ions to the spray liquid influenced the ionization of all bile acids, specifically enhancing the ionization efficiency of unconjugated bile acids.

Keywords: Bile acid; ESI; Ionization efficiency; CID; Intra-molecular hydrogen bonding

1. Introduction

The combination of electrospray ionization (ESI) with high-performance liquid chromatography provides the most suitable ionization method for highly sensitive mass spectrometric analysis of thermolabile hydrophilic substances, such as macromolecules [1,2], drugs and drug metabolites [3–5]. This method gently produces gas-phase ions without the need for heating, impact with particles, or energy irradiation [6]. Application of high voltages, usually of several kV, to the tip of the thin capillary tube produces both positively and negatively charged regions within the aqueous solution, generating fine charged droplets in the electrospray that travel toward the opposite electrode. The formed charged droplets become progressively smaller, until finally the charge is transferred to analytes in the gas-phase. Both the physico-chemical properties of analytes and the components of the solution within the fine droplets influence the ionization efficiency. We investigated the ionization behavior of unconjugated, glycine-conjugated and taurine-conjugated bile acid 3-sulfates in the presence of three different organic anions, and reported the effect of their pK_a on the peak intensity of doubly charged ions in comparison to that seen for singly charged ions [7].

In the present study, to optimize the ionization conditions suitable for liquid chromatography/tandem mass spectrometric analysis of bile acids and their glycine and taurine conjugates (Fig. 1), we investigated the effect on ionization efficiency of organic anions as additives to the spray liquid. The relationship between ionization efficiency and the structure of the bile acids depended on intra-molecular hydrogen bonding, as revealed in full scan ESI mass spectra mode from the product ion spectra derived using deprotonated molecules as precursor ions.

2. Experimental

2.1. Reagents

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Glycine (G) and taurine-conjugated (T) bile acids and

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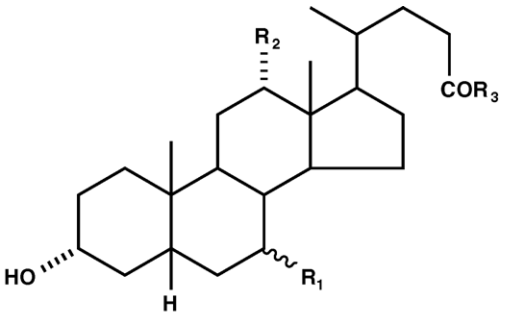

	R_1	R_2
cholic acid	α-OH	OH
chenodeoxycholic acid	α-OH	H
deoxycholic acid	H	OH
ursodeoxycholic acid	β-OH	H
lithocholic acid	H	H

	R_3
unconjugate	OH
glycine conjugate	$NHCH_2COOH$
taurine conjugate	$NH(CH_2)_2SO_3H$

Fig. 1. Structure of bile acids used in this study.

dehydroepiandrosterone 3-sulfate (DHEA-3S) were synthesized in our laboratory as previously reported [8–11]. Additional reagents and solvents were of HPLC grade.

2.2. Measurement of mass spectra of bile acids

Unconjugated, glycine-conjugated and taurine-conjugated bile acids were dissolved at 1 nmol/mL in five solvents: water/methanol (3:7, v/v), 20 mM ammonium acetate in water/methanol (3:7, v/v), 20 mM acetic acid in water/methanol (3:7, v/v), 20 mM formic acid in water/methanol (3:7, v/v) and 20 mM trifluoroacetic acid (TFA) in water/methanol (3:7, v/v). Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was performed using a QSTAR XL apparatus (Applied Biosystems). Bile acids were analyzed in infusion mode at a flow rate of 5 μL/min. All ESI mass spectra were measured at a scan range from *m/z* 10 to 2000 with a spray voltage of −4800 V and a declustering potential of −55.0 V. The product ion scan was performed using a collision energy ranging from 40.0 to 65.0 eV and a collision gas constant of three units (N_2).

3. Results and discussion

3.1. Effect of organic anions on bile acid ionization

The ionization of small ionic molecules is significantly influenced by organic solvents and organic anions [12,13]. To standardize the observed effects of various experimental factors, we normalized our results using DHEA-3S as an internal standard. The intensities of all bile acids relative to DHEA-3S are listed in Table 1. In a neutral or weakly acidic solution, the relative intensities of all bile acids were not altered. In ESI, the application of a high negative voltage to the tip of the capillary produces a large number of fine anion-rich droplets in the gas-phase. Although the droplets include water, organic solvents and organic anions, the principal negative ion contained in the droplets is hydroxide ion. Therefore, the addition of acetic acid and formic acid did not significantly affect the intensity of the deprotonated molecules. The addition of TFA, however, greatly influenced the intensity of all bile acids. No deprotonated peaks representing unconjugated bile acids were seen in 20 mM TFA in water/methanol (3:7, v/v). For glycine conjugates, the addition of TFA to the spray liquid resulted in the reduction of the peak intensity. In contrast, TFA addition increased the intensity of taurine-conjugated bile acids. Although the high electrical conductivity of spray liquid does not have a salutary effect on electrospray ionization, the proximity of the pK_a of TFA (0.23) to that of taurine (0.3) may lead to this previously reported result [7].

Table 1
Relative intensity of bile acids in ESI process

	a	b	c	d	e
Unconjugated					
CA	0.104	0.079	0.089	ND	0.502
CDCA	0.164	0.150	0.051	ND	0.590
DCA	0.102	0.143	0.085	ND	0.561
UDCA	0.060	0.035	0.027	ND	0.187
LCA	0.094	0.075	0.034	ND	0.283
Glycine-conjugated					
CA	0.349	0.255	0.380	0.093	0.993
CDCA	0.268	0.261	0.167	0.018	0.716
DCA	0.263	0.305	0.245	0.097	0.734
UDCA	0.760	0.522	0.702	0.059	1.754
LCA	0.403	0.291	0.359	0.012	0.846
Taurine-conjugated					
CA	0.851	0.867	1.009	2.759	1.382
CDCA	0.390	0.531	0.291	1.265	0.654
DCA	0.403	0.588	0.466	1.526	0.721
UDCA	0.580	0.595	0.714	1.725	0.904
LCA	0.424	0.512	0.534	1.196	0.683

a: water/methanol (3:7, v/v), b: water/methanol (3:7, v/v) containing 20 mM acetic acid, c: water/methanol (3:7, v/v) containing 20 mM formic acid, d: water/methanol (3:7, v/v) containing 20 mM trifluoroacetic acid, e: water/methanol (3:7, v/v) containing 20 mM ammonium acetate, ND: not detected.

CDCA (3α,7α-dihydroxycholanoic acid) and UDCA (3α,7β-dihydroxycholanoic acid), both possess a weak acidic group at C-24. The intensity of the deprotonated ions of these unconjugated dihydroxy bile acids were similar to each other in both neutral and weak acidic solutions. The intensities of CA (3α,7α,12α-trihydroxycholanoic acid) and DCA (3α,12α-dihydroxycholanoic acid) were 2.5-fold higher in water/methanol (3:7, v/v) containing 20 mM formic acid than in water/methanol (3:7, v/v) alone (Fig. 2). Both of

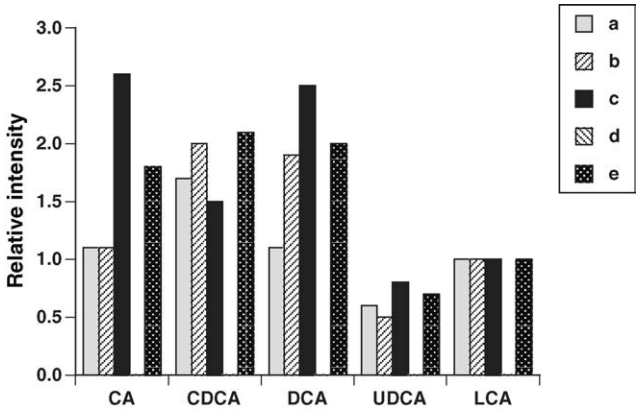

Fig. 2. The effect of spray liquid composition on the intensity of deprotonated unconjugated bile acid derivatives relative to lithocholic acid. a, Water/methanol (3:7, v/v); b, water/methanol (3:7, v/v) containing 20 mM acetic acid; c, water/methanol (3:7, v/v) containing 20 mM formic acid; d, water/methanol (3:7, v/v) containing 20 mM trifluoroacetic acid; e, water/methanol (3:7, v/v) containing 20 mM ammonium acetate.

these bile acids have a 12α-hydroxyl group on the steroid skeleton, which can participate in intra-molecular hydrogen bonding with a carboxyl group at the C-24 position. This hydrogen bonding generates the characteristic chromatographic behavior of 12α-hydroxylated bile acids [7]. Intra-molecular hydrogen bonding between the 12α-hydroxyl and carboxyl groups may lead to stronger localization of the electron on the carboxyl group, accelerating the electrospray ionization of CA and DCA.

3.2. Effect of ammonium cation on bile acid ionization

All bile acids had a higher intensity in 20 mM ammonium acetate solution than in 20 mM acetic acid (Table 1). While this tendency was clear for unconjugated and glycine-conjugated bile acids, the increase in intensity in ammonium acetate was weaker for taurine conjugates. This result demonstrates the effect of side chain ionic group acidity on ionization efficiency. These two solutions have different pHs: the pH of 20 mM ammonium acetate in water/methanol (3:7, v/v) is approximately 6–7, similar to a solution without any organic anion additives. The peak intensity in 20 mM ammonium acetate in water/methanol (3:7, v/v) was 1.5- to five-fold greater than that seen in water/methanol alone (3:7, v/v), indicating the contribution of ammonium ions to the increase in intensity seen using ammonium acetate solution.

Negative charged ions, such as hydroxide and acetic ions, and a small amount of ammonium ion exist within the charged fine droplets. Although some of these ions become neutral molecules to escape as vapor from the droplets, an ammonium ions, a positively charged ion, may also accelerate to dissociate analytes within the fine droplets into an anion, which serves as counter ions for the ammonium. Therefore, the presence of ammonium ions may cause the analyte to leave as an anion in the subdivided charged droplets, contributing to improved ionization efficiency of bile acids.

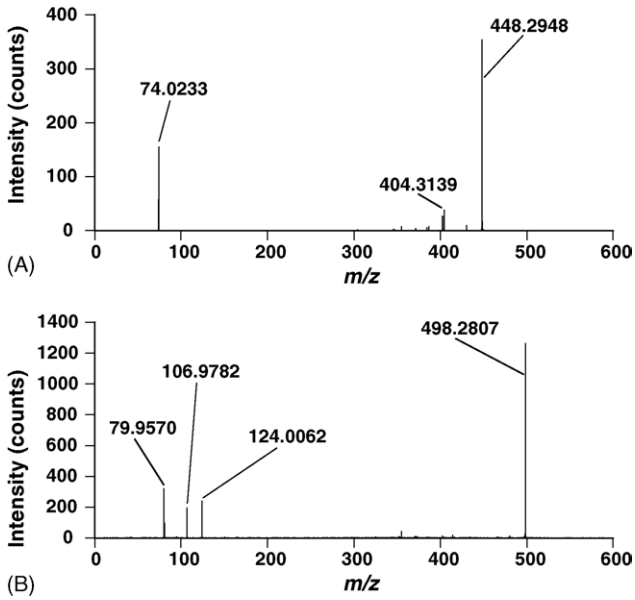

Fig. 3. Product ion mass spectra of (A) glycine-conjugated deoxycholic acid and (B) taurine-conjugated deoxycholic acid. Conditions: instrument, QSTAR XL; flow rate, 5 μL/min; electrospray voltage, −4800 V; declustering potential, −55.0 V; collision energy, (A) 40.0 eV, (B) 65.0 eV; collision gas, 3 unit (N_2).

3.3. Fragmentation of amino acid-conjugated bile acids in low energy-CID

Bile acids have hydroxyl groups on the steroid skeleton and an anionic functional group on the side chain. In positive ion detection mode, hydroxyl groups were easily eliminated from bile acid molecules in low energy-CID [14,15]. Negative charge localizes to the carboxylic acid moiety; therefore, the negatively charged product ions are derived from the side chain. Unconjugated bile acids did not generate any effective product ions in low energy-CID, because of the high stability of these molecules. Glycine-conjugated bile acids produced a product ion at *m*/*z* 74 (Fig. 3), a $NH_2CH_2COO^-$ derived from the glycine moiety. Taurine-conjugated bile acids produced three product ions at *m*/*z* 80, *m*/*z* 107 and *m*/*z* 124, corresponding to the SO_3^-, $CH_2CHSO_3^-$ and $NH_2CH_2CH_2SO_3^-$ derivatives of the taurine moiety, respectively. The relative intensities for the product ions in comparison to the precursor ions (Table 2) for the *m*/*z* 74 product ion conformed to the following relationship: GLCA (3α-hydroxy) > GUDCA (3α,7β-dihydroxy) = GCDCA (3α,7α-dihydroxy) > GDCA (3α,12α-

Table 2
Relative intensity of product ions derived from glycine- or taurine-conjugated bile acids

	Glycine conjugates	Taurine conjugates		
	m/*z* 74	*m*/*z* 80	*m*/*z* 107	*m*/*z* 124
CA	33.68	15.35	10.22	13.83
CDCA	56.97	23.60	11.86	11.86
DCA	43.79	25.14	15.46	18.95
UDCA	58.68	29.49	14.07	13.87
LCA	84.15	39.02	19.70	16.28

dihydroxy) > GCA (3α,7α,12α-trihydroxy). For taurine conjugates, the most intense product ion was *m*/*z* 80; the intensity was approximately twice that of the *m*/*z* 107 and *m*/*z* 124 ions for TCDCA, TUDCA and TLCA. For TCA and TDCA, however, the intensity ratio of the product ion at *m*/*z* 124 was greater than that at *m*/*z* 80. These phenomena may arise from differences in the stereostructure of each bile acid. The hydroxyl group at the C-12α position can form hydrogen bonds with both an oxygen atom at C-24 and the carboxyl and sulfonyl groups at the end of the side chain. Although intra-molecular hydrogen bonding between the 12α-hydroxyl group and the acidic functional group on the side chain contributed to ionization during the ESI process, these interactions inhibited the production of product ions at *m*/*z* 74 for glycine conjugates and at *m*/*z* 80 for taurine conjugates during low energy-CID. Intra-molecular hydrogen bonding may stabilize an anionic compound as a gas-phase ion in the CID chamber.

4. Conclusions

In this study, we investigated the effect on ionization efficiency of organic anions as additives to the spray liquid. Organic acids significantly influenced ionization efficiency; the use of stronger acids reduced the peak intensity of a deprotonated molecule for both unconjugated and glycine-conjugated bile acids. For taurine conjugates, the use of TFA, the strongest acid examined, improved the peak intensity. A hydroxyl group at the C-12α position in CA and DCA easily forms intra-molecular hydrogen bonds with a carboxyl group at the end of the side chain. This interaction accelerates ionization. Intra-molecular hydrogen bonding may also affect the generation of product ions from a deprotonated molecule serving as a precursor ion in low energy-CID. The addition of ammonium ion into the spray liquid influenced the ionization of all bile acids, which was most pronounced for unconjugated bile acids. The investigation of ionization efficiency of the target substances is very important for development of the high sensitive and reproducible analytical method by LC/MS, and addition of suitable concentration of ammonium salt is useful for increase of sensitivity of anionic compounds on ESI-MS.

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