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High-performance ion-pair chromatographic behaviour of conjugated bile acids with di-*n*-butylamine acetate

Tomoaki Sasaki[a], Takashi Iida[a,*], Toshio Nambara[b]

[a]*Department of Chemistry, College of Humanities & Sciences, Nihon University, Setagaya, Sakurajousui, Tokyo 156-8550, Japan*
[b]*Hoshi University, Shinagawa, Ebara, Tokyo 147-8501, Japan*

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Abstract

This paper dealt with a simple and efficient method for separating a mixture of different series of ionic, high polar, and hydrophilic conjugates of bile acids by high-performance ion-pair chromatography (HPIPC) with a new volatile ion-pair chromatographic reagent, di-*n*-butylamine acetate (DBAA), as a mobile phase additive. The substrates examined included eleven different classes of C-24 glycine- or taurine-amidated, 3-sulfated, 3-glucosylated, 3-*N*-acetylglucosaminidated, and 3-glucuronidated conjugates of cholic, chenodeoxycholic, urosodeoxycholic, and deoxycholic acids, as well as their double-conjugated forms. The anionic conjugated bile acids were chromatographed on a C_{18} reversed-phase ion-pair column, eluting with methanol–water (65:35, v/v) containing 5 m*M* of DBAA as a counter ion. Satisfactory chromatographic separation and column performance were attained by DBAA, compared with conventionally used non-volatile tetra-*n*-butylammonium phosphate. The present HPIPC method with DBAA provides an insight into the separation and structural elucidation of these biologically important bile acid conjugates and may be proved to be applied to HPLC–mass spectrometric analysis.

Keywords: Ion-pairing reagents; Bile acids; Dibutylamine acetate; Tetrabutylammonium phosphate

1. Introduction

Bile acids are biosynthesized from cholesterol in liver and their composition and concentration in biological materials are closely related to hepatobiliary diseases. The large majority of naturally occurring bile acids present as the conjugated forms at C-24 carboxyl and/or C-3 hydroxyl groups with glycine, taurine, sulfonic acid, D-glucose, *N*-acetyl-D-glucosamine or D-glucuronic acid, as well as their double-conjugates. Reversed-phase (RP) high-performance liquid chromatography (HPLC) should represent a method of choice for the assay of such ionic, high polar and hydrophilic conjugates of bile acids, because it is able to perform direct analysis of these compounds without prior deconjugation and subsequent suitable derivatization [1–3]. Therefore, a number of RP–HPLC behaviours and separations have been reported independently for each homologous series of glycine- and taurine-amidated, sulfated, glucosidated, *N*-acetylglucosaminidated, and glucronidated conjugates and their double-conjugated forms of prominent bile acids, cholic (CA), chenodeoxycholic (CDCA), ursodeoxycholic

*Corresponding author. Tel.: +81-3-3329-1151; fax: +81-3-3303-9899.

E-mail address: takaiida@chs.nihon-u.ac.jp (T. Iida).

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(UDCA), deoxycholic (DCA), and lithocholic (LCA) acids [4–6]. According to those papers, peak shapes, separation efficiency, and elution sequence of the respective conjugates were strongly influenced, particularly by the pH of a buffer solution of the aqueous organic mobile phase. Hence, simultaneous HPLC analysis of a complicated mixture of the different series of conjugated bile acids present in biological fluids is severely limited without prior group fractionation based on the conjugation modes [1].

Meanwhile, ionic and hydrophilic compounds which are much less retained on a RP column appear to be best analyzed by means of some modifications of the measuring procedure. One of the choice of the procedure is high-performance ion-pair chromatography (HPIPC). For this purpose, several groups of investigators have studied the optimal conditions and retention behaviour of C-24 glycine- and taurine-amidatated bile acids by HPIPC with tetra-*n*-butylammonium phosphate (TBAP) as an ion-pair chromatographic reagent (IPCR) [7–12]. Although HPIPC with TBAP of the C-24 amidated bile acids so far examined has several advantages, one of which is that it does not have the strict pH control problem of the mobile phase, depending upon the conjugation forms of the substrates, the method received much less attention, probably owing to limited applicability, comparatively rapid deterioration of column performance, and/or the non-volatile nature of TBAP.

Recently, a new type of a highly volatile IPCR, di-*n*-butylamine acetate (DBAA), for the use of an online combination of HPLC with mass spectrometry (MS) has been developed. The catalogue (TCI-Ace, A5702) from the manufacturer (Tokyo Kasei Kogyo, Tokyo, Japan) has recommended the use of this new IPCR, because of its high volatility, which is especially well suited for the HPIPC–MS application. The successful use of DBAA for the HPLC–MS separation and characterization of ionic sulfonate salts prompted us to apply this agent in the HPIPC analysis of conjugated bile acids. This paper describes an extension of the usefulness of DBAA and HPIPC behaviour of various series of single- and double-conjugated bile acids induced by DBAA as a mobile phase additive.

2. Experimental

2.1. Samples and Reagents

0.5 mol l^{-1} DBAA solution $\{[CH_3(CH_2)_3]_2NH\cdot CH_3COOH\}$ and 0.5 mol l^{-1} TBAP solution $\{[CH_3(CH_2)_3]_4NH_2PO_4\}$ were available from Tokyo Kasei Kogyo and Wako (Osaka, Japan), respectively. All other reagents and solvents were of analytical-reagent grade and used without further purification.

Forty-three conjugated bile acids, which are classified into eleven groups according to the conjugation forms, were examined and their abbreviations used in this study are as follows: (1) Glycine-conjugated bile acids=Glyco-BA: Glycocholic acid=Glyco-CA (**1**); glycochenodeoxycholic acid=Glyco-CDCA (**2**); glycoursodeoxycholic acid=Glyco-UDCA (**3**); glycodeoxycholic acid=Glyco-DCA (**4**). (2) Taurine-conjugated bile acids (as the sodium salts)=Tauro-BA: Taurocholic acid=Tauro-CA (**5**); taurochenodeoxycholic acid=Tauro-CDCA (**6**); tauroursodeoxycholic acid=Tauro-UDCA (**7**); taurodeoxycholic acid=Tauro-DCA (**8**). (3) Bile acid 3-sulfate (as the sodium salts)=BA 3-Sul: Cholic acid 3-sulfate=CA 3-Sul (**9**); chenodeoxycholic acid 3-sulfate=CDCA 3-Sul (**10**); ursodeoxycholic acid 3-sulfate=UDCA 3-Sul (**11**); deoxycholic acid 3-sulfate=DCA 3-Sul (**12**). (4) Glycine-conjugated bile acid 3-sulfates (as the disodium salts)=Glyco-BA 3-Sul: Glycocholic acid 3-sulfate=Glyco-CA 3-Sul (**13**); glycochenodeoxycholic acid 3-sulfate=Glyco-CDCA 3-Sul (**14**); glycoursodeoxycholic acid 3-sulfate=Glyco-UDCA 3-Sul (**15**); glycodeoxycholic acid 3-sulfate=Glyco-DCA 3-Sul (**16**). (5) Taurine-conjugated bile acid 3-sulfates (as the disodium salts)=Tauro-BA 3-Sul: Taurocholic acid 3-sulfate=Tauro-CA 3-Sul (**17**); taurochenodeoxycholic acid 3-sulfate=Tauro-CDCA 3-Sul (**18**); tauroursodeoxycholic acid 3-sulfate=Tauro-UDCA 3-Sul (**19**); taurodeoxycholic acid 3-sulfate=Tauro-DCA 3-Sul (**20**). (6) Glycine-conjugated bile acid 3-glucoside=Glyco-BA 3-Glc: Glycocholic acid 3-glucoside=Glyco-CA 3-Glc (**21**); glycochenodeoxycholic acid 3-glucoside=Glyco-CDCA 3-Glc (**22**); glycoursodeoxycholic acid 3-glucoside=Glyco-UDCA 3-Glc (**23**); glycodeoxycholic acid 3-glucoside=Glyco-DCA 3-Glc (**24**). (7) Taurine-conjugated bile acid 3-glucoside (as the

sodium salts)=Tauro-BA 3-Glc: Taurocholic acid 3-glucoside=Tauro-CA 3-Glc (**25**); taurochenodeoxycholic acid 3-glucoside=Tauro-CDCA 3-Glc (**26**); tauroursodeoxycholic acid 3-glucoside=Tauro-UDCA 3-Glc (**27**); taurodeoxycholic acid 3-glucoside=Tauro-DCA 3-Glc (**28**). (8) Bile acid 3-*N*-acetylglucosaminide=BA 3-GlcNAc: Cholic acid 3-*N*-acetylglucosaminide=CA 3-GlcNAc (**29**); chenodeoxycholic acid 3-*N*-acetylglucosaminide=CDCA 3-GlcNAc (**30**); ursodeoxycholic acid 3-*N*-acetylglucosaminide=UDCA 3-GlcNAc (**31**); deoxycholic acid 3-*N*-acetylglucosaminide=DCA 3-GlcNAc (**32**). (9) Glycine-conjugated bile acid 3-*N*-acetylglucosaminides=Glyco-BA 3-GlcNAc: Glycocholic acid 3-*N*-acetylglucosaminide=Glyco-CA 3-GlcNAc (**33**); glycochenodeoxycholic acid 3-*N*-acetylglucosaminide=Glyco-CDCA 3-GlcNAc (**34**); glycodeoxycholic acid 3-*N*-acetylglucosaminide=Glyco-DCA 3-GlcNAc (**35**). (10) Taurine-conjugated bile acid 3-*N*-acetylglucosaminides (as the sodium salt)=Tauro-BA 3-GlcNAc: Taurocholic acid 3-*N*-acetylglucosaminide=Tauro-CA 3-GlcNAc (**36**); taurochenodeoxycholic acid 3-*N*-acetylglucosaminide=Tauro-CDCA 3-GlcNAc (**37**); tauroursodeoxycholic acid 3-*N*-acetylglucosaminide=Tauro-UDCA 3-GlcNAc (**38**); taurodeoxycholic acid 3-*N*-acetylglucosaminide=Tauro-DCA 3-GlcNAc (**39**). (11) Bile acid 3-glucuronides=BA 3-GlcA: Cholic acid 3-glucuronide=CA 3-GlcA (**40**); chenodeoxycholic acid 3-glucuronide=CDCA 3-GlcA (**41**); ursodeoxycholic acid 3-glucuronide=UDCA 3-GlcA (**42**); deoxycholic acid 3-glucuronide=DCA 3-GlcA; (**43**). Almost all of the conjugated bile acid samples were from our laboratory collection synthesized in these laboratories [13–17].

2.2. Chromatography

The apparatus used was a Jasco Gulliver Series HPLC (PU-980 intelligent pump, HG-980-31 solvent mixing module, and DG-980-50 degasser; Tokyo, Japan) equipped with a Shimadzu SPD-6A ultraviolet detector and a Chromatopac C-R6A data-processing system (Kyoto, Japan). Detection was at 205 nm and 0.08 absorbance units of full scale (AUFS). Each of the bile acid samples (ca. 0.4 mg) was dissolved in eluent (0.6 ml), the solution was filtered through a 0.45 μm Sumplep filter (Millipore, Bedford, MA, USA) and an aliquot (10~15 μl) of the sample solution was injected into the HPLC system together with an internal standard.

An Ultrasphere IP column (25 cm×4.6 mm I.D.; Beckman Instruments, CA, USA) was used under ambient conditions. Mixtures of methanol–water–0.5 mol l^{-1} DBAA (65:35:1, v/v/v; pH 7.8) and methanol–water–0.5 mol l^{-1} TBAP (70:30:1, v/v/v; pH 6.3) were used as aqueous organic mobile phases for the isocratic condition at a flow-rate of 1 ml/min. The separation was performed at the column temperature of 30°C.

3. Results and discussion

In order to clarify and verify the usefulness of DBAA as a new volatile IPCR in HPIPC, as well as HPLC–MS, the chromatographic behaviour and separation were undertaken for the eleven series of 43 single- and double-conjugates of bile acids. Simultaneous analysis of these compounds in conventional RP–HPLC is usually difficult, because of their large differences in the specific physicochemical properties. Thus, although unconjugated and conjugated bile acids are amphipathic compounds possessing both hydrophilic (or polar) and lipophilic (or non-polar) moieties in the same molecules, the degree of the dissociations in a solution varies to a large extent depending upon the conjugation forms [4]; i.e.,unconjugates, pK_a=ca. 6.0; glycine conjugates, pK_a=ca. 4.5; taurine conjugates, pK_a=ca. 1.5; glucuronide conjugates, pK_a=ca. 3.5. Such a wide range of the differences in the pK_a values requires the problem of precise pH control of the mobile phase, when a simultaneous RP–HPLC analysis is carried out with a mixture of the different series of the conjugated bile acids.

It is also generally accepted that in conventional RP–HPLC the retention behaviour can be explained in terms of the differences in the affinity of substrates against hydrophilic aqueous organic mobile phase or lipophilic C_{18}-bonded alkyl stationary phase [4]. Introduction of a lipophilic substituent or bonding of a lipophilic counter ion into the substrates enhances the affinity with the stationary phase,

which results in easy control of the retentions only by reducing the water content in the mobile phase.

On the other hand, the most important feature in HPIPC is that the problem of strict pH control in RP-HPLC is eliminated. The addition of cationic DBAA to the mobile phase combines with anionic bile acid species, affording the corresponding paired-ion substrates [7]. For example, anionic tauro-BA conjugates (**5**–**8**) and di-*n*-butylammonium cation form the ion-pairings [$-CH_2SO_3^- \cdot {}^+NH_2(C_4H_9)_2$], which enhance considerably the lipophilicity of the substrate molecules, and thereby the C_{18} stationary phase retains the paired-ion substrates much more effectively. The lipophilic nature of the resulting paired-ion substrates may enable us to carry out the resolution of ''difficult-to-separate'' pairs.

The so-called ''hyphenated chromatographic analyzer'' represented by HPLC–MS equipped with a fast atom bombardment, thermospray or electrospray ionization interface have recently been introduced to the separation and structural determination of some conjugated bile acids [2,3,18–20]. In particular, a main advantage of HPLC–MS is the fact that ionic, high polar, and hydrophilic compounds can be analyzed directly without need for prior deconjugation and a suitable derivatization. In addition, HPLC–MS offers a means of enhancing the sensitivity and specificity of individual components in bile acid mixtures. These characteristics provide valuable informations concerning the site, type, and/or number of conjugations in naturally occurring bile acids. However, there still a need for several improvements and restrictions in the widespread application of HPLC–MS. One of the main obstacles to be addressed in utilising the instruments is the less or non-volatility of an excess amount of a buffer species or an IPCR in the mobile phase.

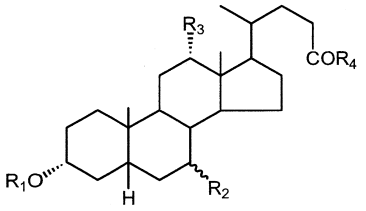
As far as we are aware, previous papers on the HPIPC of bile acids have dealt only with C-24 glycine- and taurine-BA conjugates (**1**–**8**) by the use of non-volatile TBAP as an IPCR [7–12]. Although the addition of TBAP in the mobile phase provided a clean separation of those compounds, it has several drawbacks: TBAP leads to rapid deterioration of column performance and is inadequate for an interface in HPLC–MS due to the lack of volatility. Therefore, the development of a new volatile IPCR, such as DBAA, is urgently required. On the basis of the above mentioned facts and problems, subsequent experiments were carried out in this study.

The chemical structures of individual compounds examined in this study are shown in Fig. 1. They are classified into the C-24 glycine- and taurine-amidated, 3-sulfated, 3-glucosylated, 3-*N*-acetylglucosaminidated, and 3-glucuronidated conjugates, as well as their double-conjugated forms, of four prominent naturally occurring bile acids, i.e., CA, CDCA, UDCA, and DCA.

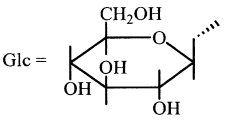
Fig. 2 shows the effect of the DBAA concentration in the eluent (methanol–water, 75:25, v/v) on the separation of tauro-BA mixtures (**5**–**8**) on a C_{18} Ultrasphere IP column. The four compounds, which were only faintly retained on the column and came out together in the absence of DBAA, were resolved completely by adding an increasing amount (0~10 m*M*) of DBAA. In this study, the eluate containing 5 m*M* of DBAA was chosen, since the addition of a large excess amount of DBAA is supposed to render the column shorten its lifetime (see below) [7] and to exert a possible inhibitory action on a HPLC–MS interface.

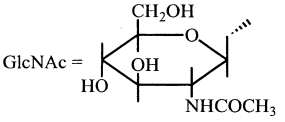
Table 1 compiles the retention data of all of the forty-three bile acid conjugates examined on a C_{18} Ultrasphere IP column, eluting with a mixture of methanol–water (65:35, v/v) containing 5 m*M* of DBAA as a mobile phase modifier. For the purpose of comparison, the table also contains the data obtained by adding commonly used TBAP as an IPCR. The retention data were expressed in terms of the retention factor (k') and the relative retention factor (rk'; relative to glyco-CDCA **2**). Under the HPIPC conditions examined, chromatographic peaks with good shape and separation were generally achieved for respective compounds not only in homologous series but also in different groups of the conjugated bile acids. Typical HPIPC chromatograms with DBAA are shown in Fig. 3.

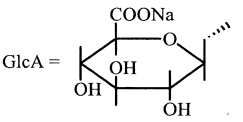
Addition of 5 m*M* of a counter ion to the mobile phase (pH 7.8) dramatically changed the elution orders of the anionic conjugates of bile acids, in comparison with those observed in the RP–HPLC under the influence of a buffer solution (pH 7.0) [4]. As expected in previous papers [8–12] of HPIPC with TBAP, tauro-BA conjugates (**5**–**9**) had larger rk' values than the corresponding glyco-BA analogs (**1**–**4**). On the other hand, taurine-amidated double-



G = -NHCH$_2$COOH , S = -SO$_3$Na , T = -NHCH$_2$CH$_2$SO$_3$Na







Glyco-BA

	R1	R2	R3	R4
1	H	α-OH	OH	G
2	H	α-OH	H	G
3	H	β-OH	H	G
4	H	H	OH	G

Tauro-BA

	R1	R2	R3	R4
5	H	α-OH	OH	T
6	H	α-OH	H	T
7	H	β-OH	H	T
8	H	H	OH	T

BA 3-Sul

	R1	R2	R3	R4
9	S	α-OH	OH	OH
10	S	α-OH	H	OH
11	S	β-OH	H	OH
12	S	H	OH	OH

Glyco-BA 3-Sul

	R1	R2	R3	R4
13	S	α-OH	OH	G
14	S	α-OH	H	G
15	S	β-OH	H	G
16	S	H	OH	G

Tauro-BA 3-Sul

	R1	R2	R3	R4
17	S	α-OH	OH	T
18	S	α-OH	H	T
19	S	β-OH	H	T
20	S	H	OH	T

Glyco-BA 3-Glc

	R1	R2	R3	R4
21	Glc	α-OH	OH	G
22	Glc	α-OH	H	G
23	Glc	β-OH	H	G
24	Glc	H	OH	G

Tauro-BA 3-Glc

	R1	R2	R3	R4
25	Glc	α-OH	OH	T
26	Glc	α-OH	H	T
27	Glc	β-OH	H	T
28	Glc	H	OH	T

BA 3-GlcNAc

	R1	R2	R3	R4
29	GlcNAc	α-OH	OH	OH
30	GlcNAc	α-OH	H	OH
31	GlcNAc	β-OH	H	OH
32	GlcNAc	H	OH	OH

Glyco-BA 3-GlcNAc

	R1	R2	R3	R4
33	GlcNAc	α-OH	OH	G
34	GlcNAc	α-OH	H	G
35	GlcNAc	H	OH	G

Tauro-BA 3-GlcNAc

	R1	R2	R3	R4
36	GlcNAc	α-OH	OH	T
37	GlcNAc	α-OH	H	T
38	GlcNAc	β-OH	H	T
39	GlcNAc	H	OH	T

BA 3-GlcA

	R1	R2	R3	R4
40	GlcA	α-OH	OH	OH
41	GlcA	α-OH	H	OH
42	GlcA	β-OH	H	OH
43	GlcA	H	OH	OH

Fig. 1. Structures of the conjugated bile acids examined.

conjugates in each series were found to be always eluted faster than the corresponding glycine-amidated and nonamidated ones, as opposed to the single-amidated conjugates described above. For example, 3-sulfated conjugates were eluted in the order of tauro-BA 3-Sul (**17**–**20**), glyco-BA 3-Sul (**13**–**16**), and then BA 3-Sul (**9**–**12**) conjugates, which is the direct reverse to those found in RP–HPLC [4,21]. Similarly, changes in the elution order between HPIPC and RP–HPLC [5,6,22] were also

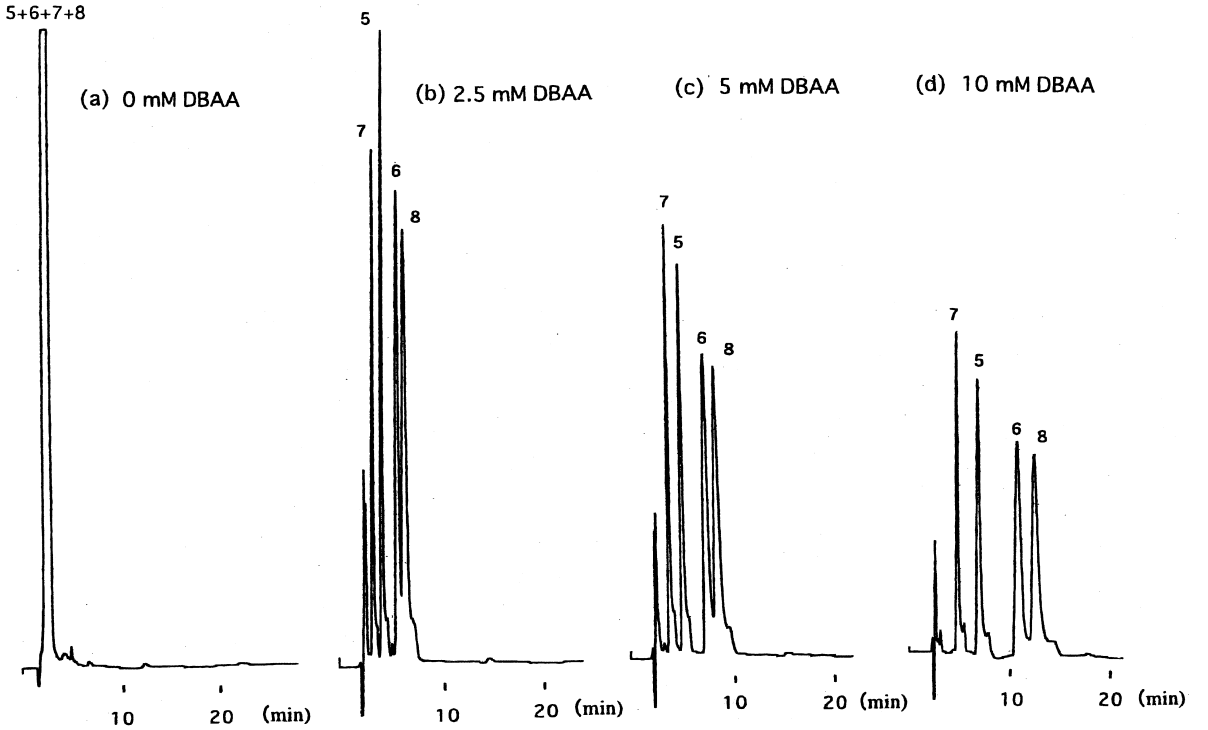


Fig. 2. Effect of DBAA concentration on the separation of tauro-BA mixtures in HPIPC. Conditions: column, Ultrasphere C_{18} IP column; mobile phase, methanol–water (75:25, v/v) in the absence or (b)–(d) presence of DBAA, 0.08 AUFS. Peak identification: the numbering corresponds to that in Fig. 1.

found in the following pairs of 3-glycosylated bile acids: Tauro-BA 3-Glc (**25**–**28**)<glyco-BA 3-Glc (**21**–**24**) conjugates; tauro-BA 3-GlcNAc (**36**–**39**)< glyco-BA 3-GlcNAc (**33**–**35**)<BA 3-GlcNAc (**29**–**32**) conjugates; BA-GlcA (**40**–**43**)<BA 3-GlcNAc (**29**–**32**) conjugates.

Irrespective of the conjugation modes, the increasing mobility of individual members in homologous series was as follows: UDCA<CA<CDCA, DCA conjugates. This elution order in HPIPC is in accord with that observed in RP–HPLC [4]. In each series, UDCA conjugates had definitely the highest mobility, because they have a hydrophilic 7β-hydroxyl group which interferes with the interaction between the hydrophobic β-surface of the 5β-steroid molecules and the hydrophobic C_{18} stationary phase, resulting in an appreciable decrease in the retentions.

The retention behaviour of two isomeric CDCA and DCA 3-glycosidic conjugates, which differ from each other in the presence of a hydroxyl at C-7 or C-12 in the aglycone moieties, is of particular interest, since it is affected by both the structures of the side-chain and sugar moiety. Thus glyco- and tauro-DCA 3-Glc conjugates (**24** and **28**) exhibit larger rk' values than the corresponding CDCA analogs (**22** and **26**), whereas the reverse is true for the nonamidated and amidated CDCA and DCA 3-GlcNAc conjugates (**30** versus **32**, **34** versus **35**, and **37** versus **39**). Analogous nonamidated CDCA and DCA 3-GlcA conjugates (**14** and **43**) provided almost identical rk' values. The phenomena would be ascribable the result of a combined steric interactions between the axial α-hydroxyl group at C-7 and the sugar moieties at C-3 and/or the axial α-hydroxyl group at C-12 and the terminal carboxylic or sulfonic acid residue of the side-chain [4,5].

It is also evident from Table 1 that individual compounds in the five variants of single-conjugated bile acids differing in the conjugation modes at position C-3 or C-24 were well resolved on the IP

Table 1
Retention data for single- and double-conjugated bile acids in HPIPC with DBAA or TBAP

Compound[a]		DBAA[b]		TBAP[c]	
		k'_1	rk'_1	k'_2	rk'_2
Glyco-BA					
1	Glyco-CA	4.90	0.47	3.42	0.50
2	Glyco-CDCA	10.5	1.00	6.81	1.00
3	Glyco-UDCA	2.56	0.24	1.87	0.27
4	Glyco-DCA	12.9	1.23	8.30	1.22
Tauro-BA					
5	Tauro-CA	5.30	0.51	3.63	0.53
6	Tauro-CDCA	11.5	1.10	7.32	1.08
7	Tauro-UDCA	2.74	0.26	1.87	0.27
8	Tauro-DCA	14.0	1.34	8.94	1.31
BA 3-Sul					
9	CA 3-Sul	1.72	0.16	2.00	0.29
10	CDCA 3-Sul	2.35	0.22	5.50	0.81
11	UDCA 3-Sul	0.89	0.09	0.74	0.11
12	DCA 3-Sul	2.77	0.26	6.00	0.88
Glyco-BA 3-Sul					
13	Glyco-CA 3-Sul	1.09	0.10	1.12	0.16
14	Glyco-CDCA 3-Sul	2.18	0.21	2.25	0.33
15	Glyco-UDCA 3-Sul	0.63	0.06	0.61	0.09
16	Glyco-DCA 3-Sul	2.58	0.25	2.62	0.38
Tauro-BA 3-Sul					
17	Tauro-CA 3-Sul	0.98	0.09	1.14	0.17
18	Tauro-CDCA 3-Sul	2.02	0.19	2.16	0.32
19	Tauro-UDCA 3-Sul	0.55	0.05	0.63	0.09
20	Tauro-DCA 3-Sul	2.41	0.23	2.57	0.38
Glyco-BA 3-Glc					
21	Glyco-CA 3-Glc	2.57	0.25	1.78	0.26
22	Glyco-CDCA 3-Glc	5.06	0.48	3.34	0.49
23	Glyco-UDCA 3-Glc	1.08	0.10	0.75	0.11
24	Glyco-DCA 3-Glc	6.07	0.58	4.01	0.59
Tauro-BA 3-Glc					
25	Tauro-CA 3-Glc	2.36	0.23	1.67	0.24
26	Tauro-CDCA 3-Glc	4.68	0.45	3.14	0.46
27	Tauro-UDCA 3-Glc	1.00	0.10	0.71	0.10
28	Tauro-DCA 3-Glc	5.58	0.53	3.70	0.54
BA 3-GlcNAc					
29	CA 3-GlcNAc	2.84	0.27	3.18	0.47
30	CDCA 3-GlcNAc	9.65	0.92	9.75	1.43
31	UDCA 3-GlcNAc	1.22	0.12	1.63	0.24
32	DCA 3-GlcNAc	6.55	0.62	7.22	1.06
Glyco-BA 3-GlcNAc					
33	Glyco-CA 3-GlcNAc	2.81	0.27	2.89	0.42
34	Glyco-CDCA 3-GlcNAc	6.96	0.66	4.21	0.62
35	Glyco-DCA 3-GlcNAc	5.33	0.51	3.25	0.48
Tauro-BA 3-GlcNAc					
36	Tauro-CA 3-GlcNAc	2.11	0.20	1.46	0.21
37	Tauro-CDCA 3-GlcNAc	6.52	0.62	4.04	0.59
38	Tauro-UDCA 3-GlcNAc	0.85	0.08	0.63	0.09
39	Tauro-DCA 3-GlcNAc	4.98	0.47	3.16	0.46
BA 3-GlcA					
40	CA 3-GlcA	1.38	0.13	2.98	0.44
41	CDCA 3-GlcA	3.48	0.33	5.42	0.80
42	UDCA 3-GlcA	0.83	0.08	1.68	0.25
43	DCA 3-GlcA	3.37	0.32	5.42	0.80

[a] For abbreviations see text.
[b] Data obtained by eluting methanol–water–0.5 mol l^{-1} DBAA. (65:35:1, v/v/v).
[c] Data obtained by eluting methanol–water–0.5 mol l^{-1} TBAP (70:30:1, v/v/v).

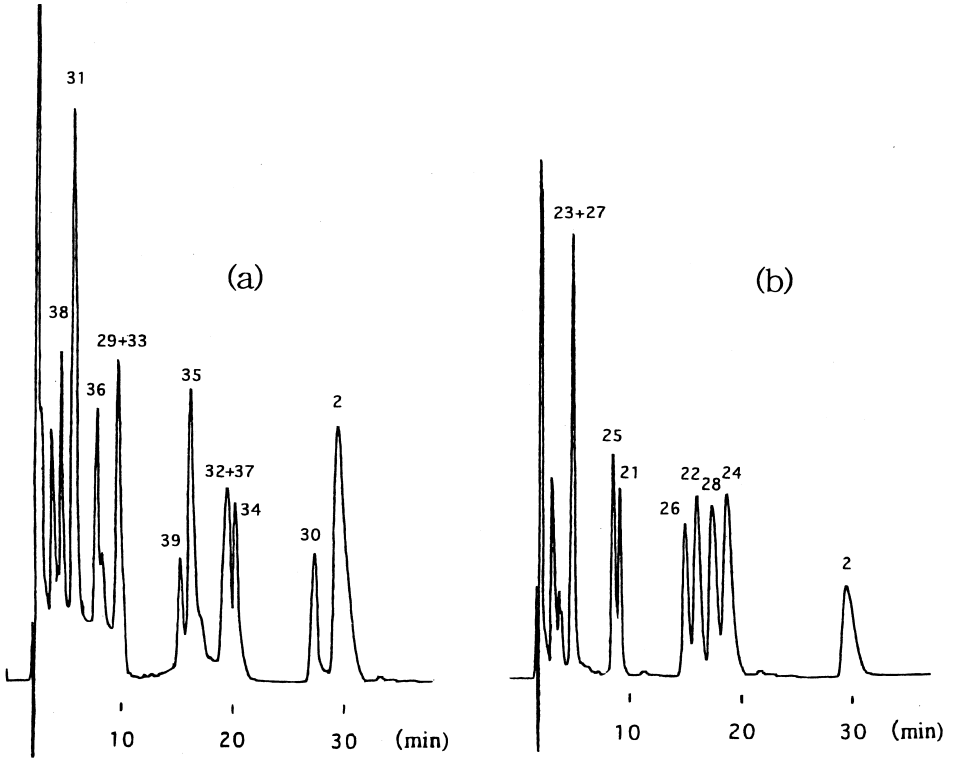


Fig. 3. Typical chromatograms of a mixture of different classes of conjugated bile acids: (a) Single- and double-conjugate GlcNAc mixtures; (b) glyco- and tauro-BA 3-Glc mixtures. Conditions: column, Ultrasphere C_{18} IP column; mobile phase, methanol–water–0.5 mol l^{-1} DBAA (65:35:1, v/v/v), 0.08 AUFS. Peak identification: the numbering corresponds to that in Fig. 1.

column with DBAA, emerging in the order of BA 3-Sul (**9**–**12**), BA 3-GlcA (**40**–**43**), BA 3-GlcNAc (**29**–**42**), glyco-BA (**1**–**4**), and then tauro-BA (**5**–**8**) conjugates. Based on the thin-layer chromatographic mobility on both normal-phase and RP plates [23], approximate polarity of the ionic conjugated bile acids would be deduced to be decreased in the order of tauro-BA, BA 3-Sul, BA 3-GlcA, and glyco-BA conjugates. The result indicates that DBAA does influence most significantly the retention of the tauro-BA conjugates, while the retarding effect of DBAA on the retention of the BA 3-Sul conjugates is much smaller than the expected. Such an interesting behaviour can, therefore, be usefully utilized for differentiating between the sulfonic acid group (as sodium sulfonate, $-SO_3Na$) at the side-chain in **5**–**8** and the sulfate ester group (as sodium sulfate, $-OSO_3Na$) at C-3 in **9**–**12**.

The following general trend of the increasing rk' values was observed for the double-conjugated bile acids: tauro-BA 3-Sul (**17**–**20**)<glyco-BA 3-Sul (**13**–**16**)<tauro-BA 3-GlcNAc (**36**–**39**)<tauro-BA 3-Glc (**25**–**28**)<glyco-BA 3-Glc (**21**–**24**)<glyco-BA 3-GlcNAc (**33**–**35**) conjugates. Again, the tauro-BA 3-Sul, tauro-BA 3-Glc, and tauro-BA 3-GlcNAc double-conjugates always eluted before the corresponding glycine-conjugates, which are reverse to those observed in RP–HPLC [4–6]. Furthermore, while the glyco- and tauro-CDCA 3-Glc conjugates (**22** and **26**) are eluted faster than the corresponding CDCA 3-GlcNAc analogs (**34** and **37**), the reverse relationship was observed for the corresponding glyco- and tauro-DCA Glc conjugates (**24** versus **35** and **28** versus **39**).

Essentially identical HPIPC behaviours were also observed with a few exception, when the single- and double-conjugated bile acids were measured in the presence of 5 m*M* of TBAP as a mobile phase

modifier. The above alternations in the elution orders are, therefore, apparently ascribed to the differences in the affinity of more hydrophilic ionized substrates and less hydrophilic paired-ion substrates against the highly lipophilic C_{18} stationary phase.

The stability of an Ultrasphere IP column and the reproducibility of the retention data were determined continuously on different days by repeated analyses of the same stock solution of eight glyco- and tauro-BA mixtures (**1**–**8**). Fig. 4 shows the variations in the k' and rk' values of tauro-CDCA (**6**), when the initially measured k' and rk' values are defined as 100%. As can be seen it, the effect of DBAA and TBAP on the retentions and column efficiency much differed from each other. The relative standard deviations (RSDs) observed for the k' and rk' values of **6**, calculated from continuous 40 chromatograms, were 0.78 and 0.12%, respectively, for DBAA and 3.88 and 0.49%, respectively, for TBAP. Apparently, the use of non-volatile TBAP brought about some scatters in the rk' values and the sequential decreases in the k' values within a comparatively short period of time, though the deterioration of the column performance was easily regenerated by washing with water and then with methanol [7,10]. Similar trends were also found for the other C-24 amidated bile acids.

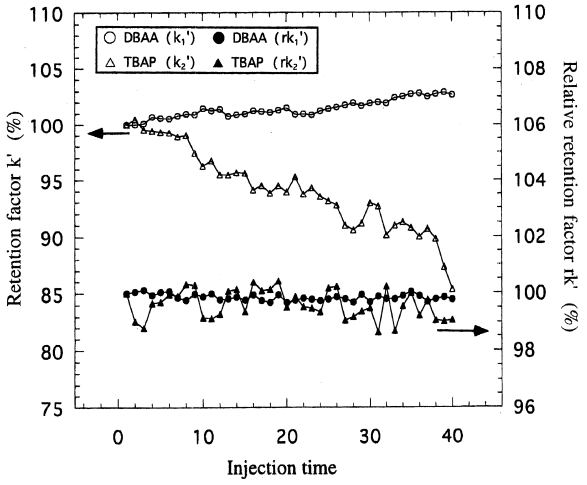


Fig. 4. Effect of DBAA and TBAP in the mobile phase on the stability of a column and the reproducibility of retention data. k'_1 and k'_2 values show the retention factors observed for tauro-CDCA (**6**) in the presence of DBAA and TBAP as mobile phase modifiers, respectively; rk'_1 and rk'_2 values were defined as the relative variation (initially injected value=100%) of k'_1 and k'_2 values of **6**, respectively.

On the other hand, no such deterioration of the column performance and decrease in the retentions with TBAP were observed for volatile DBAA which so far examined proved to be an excellent stability, reproducibility and a long lifetime of the column. The use of DBAA as an IPCR is therefore superior to that of TBAP.

The above phenomena can be probably accounted for the differences in the lipophilic character of the two IPCRs, DBAA and TBAP. Thus, C_{18}-bonded alkyl stationary phase may interact not only with a paired-ion substrate but also with an IPCR. Assuming that compared to a C_8 di-*n*-butylammonium ion, a more alkylated C_{16} tetra-*n*-butylammonium ion is accumulated gradually into the stationary phase, an effective capacity possible to interact between the paired-ion substrate and the stationary phase will be reduced.

In conclusion, the usefulness and applicability of a new volatile IPCR, DBAA, for analyzing ionized, high polar, and hydrophilic compounds by HPIPC were examined for a variety types of single- and double-conjugated bile acids. The changes in the chromatographic behaviour and the column performance induced by adding DBAA into the mobile phase were compared with those of the commonly used TBAP. The results clarified and verified that DBAA is preferable to TBAP with regard to the characteristics. The simple and efficient HPIPC method with DBAA would be usefully applied for the separation and determination of conjugated bile acids present in biological materials.

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