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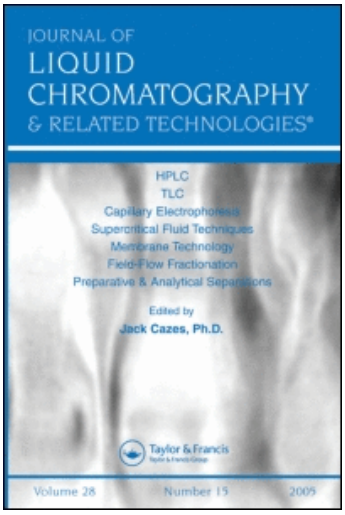

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
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To cite this Article Iida, T. , Yamaguchi, Y. , Maruyama, J. , Nishio, M. , Goto, J. and Nambara, T.(1995) 'High Performance Liquid Chromatographic Separation of Nonamidated and Glycine- and Taurine-Amidated Bile Acid 3-Glucosides', Journal of Liquid Chromatography & Related Technologies, 18: 4, 701 — 711
To link to this Article: DOI: 10.1080/10826079508009266
URL: http://dx.doi.org/10.1080/10826079508009266

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(4), 701-711 (1995)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NONAMIDATED AND GLYCINE- AND TAURINE-AMIDATED BILE ACID 3-GLUCOSIDES

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ABSTRACT

The high-performance liquid chromatographic separation of the 3-glucosides of nonamidated lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic and cholic acids, and their double conjugate forms with glycine and taurine has been carried out on a C_{18} reversed-phase column. Satisfactory separation not only of each of the three groups of the nonamidated and amidated bile acid 3-glucosides but also of the individual compounds in the same group was attained by employing acetonitrile-0.3% potassium phosphate buffer (pH, 7.0) as mobile phase. The retention data reported here provide an insight into structural elucidation of these biologically important bile acid 3-glucosides.

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INTRODUCTION

Recently, the glycosidic conjugates of bile acids are of substantiated interest in biosynthesis, metabolism, and physiological significance in connection with hepatobiliary diseases. At present, three types of glycosidic conjugation are known in bile acid metabolism in humans: glucuronidation (1,2), *N*-acetylglucos aminidation (3,4), and glucosidation (5-8). Of these glycosidic conjugates, bile acid glucosides, novel conjugates, have recently been shown to be formed in human liver microsomes by a glucosyltransferase and are preferentially excreted in human urine from patients with hepatobiliary diseases.

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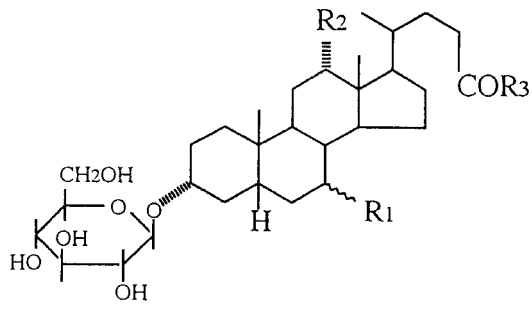
High-performance liquid chromatography (HPLC) with a reversed-phase column seems to be a most reliable method for analysis of such polar, nonvolatile and thermolabile compounds. In fact, a direct HPLC analysis of glycine-, taurine-, sulfate-, glucuronide- and *N*-acetylglucosaminide-conjugated bile acids without prior deconjugation has been successfully applied (9-11).

As part of our synthetic program on potential bile acid metabolites, we have recently synthesized a series of nonamidated and glycine- and taurine-amidated bile acid 3-glucosides as authentic specimens (Fig. 1) (12). In this paper, we clarify the retention behaviors of the bile acid 3-glucosides on a reversed-phase HPLC and compare with those of analogous bile acid 3-glucuronides and 3-*N*-acetylglucosaminides reported previously (10,11).

EXPERIMENTAL

Samples and Reagents

The 3-glucosides of nonamidated and glycine- and taurine-amidated bile acids were synthesized in these



	R1	R2	R3	
1	H	H	OH	(LCA 3-Glc.)
2	α-OH	H	OH	(CDCA 3-Glc.)
3	β-OH	H	OH	(UDCA 3-Glc.)
4	H	α-OH	OH	(DCA 3-Glc.)
5	α-OH	α-OH	OH	(CA 3-Glc.)
6	H	H	$NHCH_2COOH$	(Glyco-LCA 3-Glc.)
7	α-OH	H	$NHCH_2COOH$	(Glyco-CDCA 3-Glc.)
8	β-OH	H	$NHCH_2COOH$	(Glyco-UDCA 3-Glc.)
9	H	α-OH	$NHCH_2COOH$	(Glyco-DCA 3-Glc.)
10	α-OH	α-OH	$NHCH_2COOH$	(Glyco-CA 3-Glc.)
11	H	H	$NHCH_2CH_2SO_3H$	(Tauro-LCA 3-Glc.)
12	α-OH	H	$NHCH_2CH_2SO_3H$	(Tauro-CDCA 3-Glc.)
13	β-OH	H	$NHCH_2CH_2SO_3H$	(Tauro-UDCA 3-Glc.)
14	H	α-OH	$NHCH_2CH_2SO_3H$	(Tauro-DCA 3-Glc.)
15	α-OH	α-OH	$NHCH_2CH_2SO_3H$	(Tauro-CA 3-Glc.)

FIGURE 1. Structures of bile acid 3-glucosides.

laboratories by the methods recently reported (12). All the chemicals used were of analytical reagent grade. Solvents used were of HPLC grade and were degassed by sonication prior to use.

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Apparatus

The HPLC apparatus used was a Hitachi L-6000 chromatograph (Hitachi,. Tokyo, Japan) equipped with a Shimadzu SPD-2A ultraviolet detector (Shimadzu, Kyoto, Japan); the wavelength selected for all measurements was 205 nm. A Cosmosil 5C18 column (5 μm, 150 mm X 4.6 mm I.D.) (Nacarai Tesque Inc., Kyoto, Japan) was used under ambient conditions. Acetonitrile-0.3% potassium phosphate buffer mixture (pH, 3.5~7.5; ratio from 25:75 to 35:65, v/v) was used as eluent at a flow rate of 1.0 ml/min.

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RESULTS AND DISCUSSION

The chemical structures of bile acid 3-glucosides examined in this study are shown in Fig. 1. These 3-glucosides include the derivatives of the nonamidated (1~5) and glycine- (6~10) and taurine- (11~15) amidated forms of five prominent naturally occurring bile acids [*i.e.*, lithocholic acid (LCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), and cholic acid (CA)] and differ from one another in the number, position and configuration of hydroxyl groups at positions C-3, C-7 and/or C-12 in the 5β-steroid nucleus.

Recently, Goto *et al.* have reported the HPLC behaviors of bile acid 3-glucuronides (10) and 3-*N*-acetylglucosaminides (11) on a C18 reversed-phase column using acetonitrile-potassium phosphate buffer mixture as the mobile phase and found that the capacity factor (k') of those conjugates are extensively dependent on both the structure of the substrates and the acidity of the mobile

phase. On the basis of these findings, the effect of pH of mobile phase on the k' values of each of the three groups of nonamidated and glycine- and taurine-amidated bile acid 3-glucosides were initially examined. In order to facilitate a comparison of their mobilities with those of the analogous glucuronide and *N*-acetylglucosaminide conjugates (10,11), acetonitrile-0.3% potassium phosphate buffer (pH, 3.5~7.5) was used as the eluent system. The result is expressed graphically in Fig. 2. As expected, the k' values (relative to tauro-DCA) of each compound respond to the structural differences in the 5β-steroid nucleus and C-17 side chain, and separation efficiency and analysis time of individual members of compounds are markedly influenced by pH of the mobile phase.

Among the three groups of bile acid 3-glucosides, the nonamidated forms were the most sensitive to the pH of mobile phase and then followed by glycine conjugates, and their k' values increased with decreasing pH from *ca.* 6~7. In neutral or slightly alkaline condition (pH, 7.0~7.5), each of these two groups of compounds exhibited a well-shaped peak with a relatively small k' value and short retention time. On the contrary, the k' values for the taurine conjugates are scarcely affected throughout the whole pH range (3.5~7.5) examined. The phenomenon is compatible with those observed with analogous bile acid 3-glucuronides (10) and 3-*N*-acetylglucosaminides (11), and can be explained in terms of the difference in the degree of dissociation in the three types of side chain (9). Based on the above data, acetonitrile-0.3% potassium phosphate buffer of pH=7.0 was chosen as suitable mobile phase.

Five nonamidated bile acid 3-glucosides differing in the number, position and configuration of hydroxyl groups in the aglycone moiety were well resolved on Cosmosil 5C$_{18}$ column using the appropriate eluent system, and the

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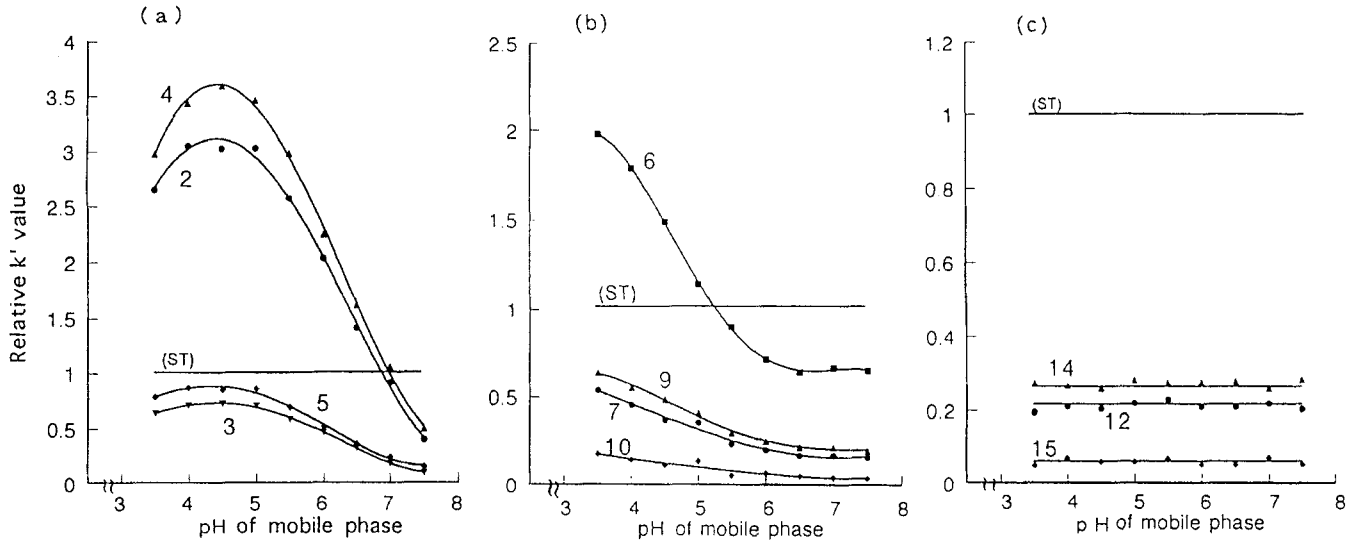

FIGURE 2. Effect of pH of mobile phase on relative k' values of(a) nonamidated and (b) glycine- and (c) taurine-amidated bile acid 3-glucosides. St (standard) = taurodeoxycholic acid. HPLC conditions: column, Cosmosil 5C18; mobile phase, acetonitrile- 0.3% potassium phosphate buffer (pH, 3.5~7.5),(30:70~35:65, v/v); flow rate, 1.0 ml/min; detection, UV at 205 nm. The numbering corresponds to that in Fig. 1.

TABLE 1. Relative k' values of nonamidated and amidated bile acid 3-glucosides on reversed-phase HPLC.[1]

Nonamidated		Glycine-amidated		Taurine-amidated	
1	2.94	6	2.37	11	3.50
2	0.84	7	0.86	12	1.26
3	0.16	8	0.16	13	0.23
4	1.00	9	1.10	14	1.52
5	0.25	10	0.29	15	0.38

[1] k' Values are expressed relative to that of DCA 3-Glc. (4); mobile phase, acetonitrile-0.3% potassium phopsphate buffer (pH, 7.0; 25:75, v/v). The numbering corresponds to that in Fig.1.

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following order of increasing mobility was observed: UDCA 3-Glc. (3)< CA 3-Glc. (5)< CDCA 3-Glc. (2)< DCA 3-Glc. (4)< LCA 3-Glc. (1). The same elution order was also observed on the corresponding glycine and taurine double conjugates. The order of mobility in each group usually corresponds to the number of hydroxyl groups in the substrates and is consistent well with those observed for the corresponding bile acid 3-glucuronides (10) and 3-N-acetylglucosaminides (11). However, the 3-glucosidic conjugates of UDCA having a β-hydroxyl group at position C-7 had decidedly the earliest mobility in each group, probably due to a decrease of the hydrophobic interaction between the β-face of the steroid nucleus and the surface of the stationary phase.

Table 1 shows the k' values [relative to DCA 3-Glc. (4)] for the 15 bile acid 3-glucosides determined on a

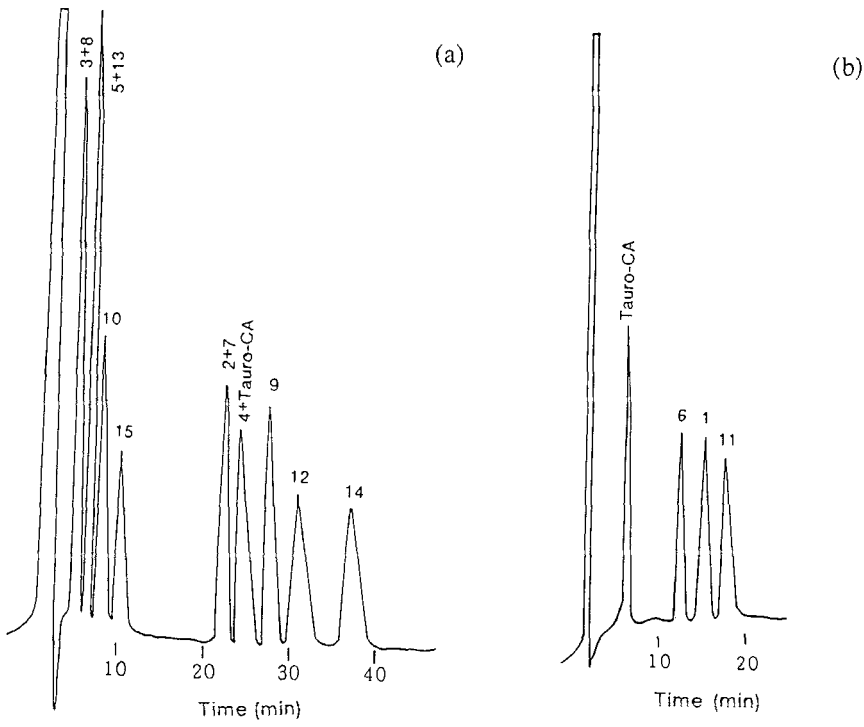

FIGURE 3. HPLC separation of a mixture of bile acid 3-glucosides. Conditions: column, Cosmosil 5C18; mobile phase, acetonitrile-0.3% potassium phosphate buffer (pH, 7.0) (a) 23:77 (v/v) and (b) 28:72 (v/v). Peak identification and the numbering as in Fig. 1.

Cosmosil 5C18 column under identical HPLC conditions [acetonitrile-0.3% potassium phosphate buffer (pH, 7.0) (25:75, v/v)]. Typical HPLC chromatograms for the separation of a mixture of these compounds are also illustrated in Fig. 3. It is not practical to analyze simultaneously the 3-glucosidic conjugates of monohydroxylated LCA (1, 6 and 11) and other di- and

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trihydroxylated bile acids in short analysis time under an isocratic condition , because of higher lipophilicity of the formers compared with the latters. In addition, the taurine conjugates in each group of bile acid 3-glucosides were found to be always eluted more slowly than the corresponding nonamidated and glycine-amidated analogs. Although two pairs of nonamidated and glycine-amidated bile acid 3-glucosides [*i.e.*, CDCA 3-Glc. (2) *vs* . glyco-CDCA 3-Glc. (7) and UDCA 3-Glc. (3) *vs* . glyco-UDCA 3-Glc. (8)] overlapped with the eluent system, they were resolved by decreasing the pH of mobile phase.

The present retention data reported here may be helpful for characterizing the structures of these biologically important bile acid 3-glucosides, and the method depends on the ability to determine simultaneously nonamidated and glycine- and taurine-amidated bile acids without prior group separation and deconjugation.

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NOTES

The following trivial names and abbreviations are used in this paper:

Lithocholic acid (LCA) = 3 α -Hydroxy-5 β -cholanoic acid

Chenodeoxycholic acid (CDCA) = 3 α ,7 α -Dihydroxy-5 β -cholanoic acid

Ursodeoxycholic acid (UDCA) = 3 α ,7 β -Dihydroxy-5 β -cholanoic acid

Deoxycholic acid (DCA) = 3 α ,12 α -Dihydroxy-5 β -cholanoic acid

Cholic acid (CA) = 3 α ,7 α ,12 α -Trihydroxy-5 β -cholanoic acid

ACKNOWLEDGMENTS

We wish to express our gratitude to Professor M.Kato, College of Engineering, Nihon University, for his continued interest and support. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Received: June 23, 1994
Accepted: October 31, 1994

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