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Method for the separation of the unconjugates and conjugates of chenodeoxycholic acid and deoxycholic acid by two-dimensional reversed-phase thin-layer chromatography with methyl β-cyclodextrin

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Abstract

A simple and efficient method for the separation of individual unconjugated bile acids and their glycine- and taurine-amidated, 3-sulfated, 3-glucosylated and 3-glucuronidated conjugates is described. The method involves the use of a two-dimensional (2D) reversed-phase (RP) high-performance thin-layer chromatographic (HPTLC) technique with methyl β-cyclodextrin (Me-β-CD). Five major unconjugated bile acids, cholic acid, chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid and lithocholic acid, and their conjugates were examined as the solutes. A high degree of separation of individual bile acids in each homologous series was achieved on a RP–HPTLC plate by developing with aqueous methanol in the first dimension and the same solvent system containing Me-β-CD in the second dimension. In particular, all of the six 'difficult-to-separate' pairs, unconjugated CDCA and DCA and their conjugated forms with glycine, taurine, sulfuric acid, D-glucose and D-glucuronic acid, were effectively resolved by adding Me-β-CD in the aqueous mobile phases with the formers having larger mobilities than the latter. The application of this 2D inclusion RP–HPLC method to the separation of glycine-conjugated bile acids in human bile is also described. The present method would be useful for separating and characterizing these bile acids present in biological materials.

Keywords: Chenodeoxycholic acid; Deoxycholic acid; Bile acids; Methyl β-cyclodextrin

1. Introduction

Major bile acids present in human biological materials are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA). In addition to the five unconjugated bile acids, conjugates on the C-24 carboxyl group with glycine or taurine and on any of the C-3, C-7 and/or C-12 hydroxyl groups with sulfuric acid, D-glucose, D-glucuronic acid or D-*N*-acetylglucosamine have been identified and characterized [1–3]. Thus, naturally occurring bile acids are constituted of a mixture of

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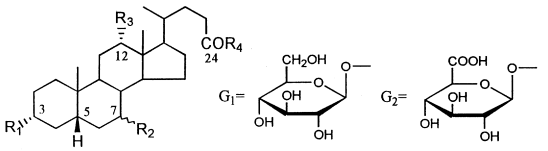
the unconjugates and conjugates of amidated, sulfated, and glycosylated bile acids. Since variations in the concentration and relative proportion of each component of the unconjugated and conjugated bile acids are closely related to hepatobiliary diseases, the group and individual separations of these compounds are important from a clinical point of view.

Of various chromatographic methods available, capillary gas chromatography (CGC) and high-performance liquid chromatography (HPLC) are now exclusively used for the profile analysis of a complicated mixture of naturally occurring bile acids, because of their high sensitivity and selectivity as well as excellent resolution efficiency [4,5]. Nevertheless, thin-layer chromatography (TLC) is still popular as routine and spot analyses of bile acids owing to its simplicity and low cost; in addition, TLC has a wide applicability which can be directly performed on comparatively polar, less volatile and thermolabile conjugated bile acids without prior deconjugation, group separation, and suitable derivatization.

In TLC analysis of bile acids, many investigators have long been confronted with a serious problem of separating two common bile acids, CDCA and DCA as well as their conjugated forms, all of which generally overlap each other [4–6]; the two dihydroxylated compounds differ only from each other in the position of hydroxyl groups in the 5β-steroid nucleus. In order to overcome the problem and/or to improve the separation, a number of TLC techniques for resolving the 'difficult' pairs have been proposed. Those include normal-phase (NP) absorption and reversed-phase (RP) partition TLC plates coated with silica gel and octadecyl (C_{18})-bonded silica gel as adsorbents, respectively [7–10]; a high-performance thin-layer chromatographic (HPTLC) plate coated with finer particle size adsorbents [11]; multiple developments of a TLC plate with the same or different solvent systems [12]; two dimensional (2D) TLC on a NP- or RP-plate in the two different directions [13,14]; a 2D TLC plate coated with both NP- and RP-adsorbents in each dimension [15]; overpressured TLC [16]. Although the separation efficiency of some of the recalcitrant pairs was enhanced by these approaches, the degree of resolution are still unsatisfactory.

Meanwhile, many studies on the effective usage of mobile phase additives or stationary phase modifiers in chromatographic sciences have recently been reported. In particular, β-cyclodextrin (β-CD) and its derivatives such as methyl β-cyclodextrin (Me-β-CD) and hydroxylpropyl β-cyclodextrin, which are toroidal-shaped cyclic oligosaccharides consisting of seven glucose units connected via α-1,4-linkage, have been shown to be effective, as they easily form stable inclusion complexes with various guest compounds [17–19]. In fact, a number of the positional, geometrical, and optical isomers of bioactive compounds have been successfully separated by adding β-CD or its analogs in aqueous mobile phases in RP–TLC or on β-CD-bonded silica-gel plates [20].

The above inclusion RP–TLC technique would be desirable for developing an improved separation of individual unconjugated bile acids and their amidated, sulfated and glycosylated ones. We report here, the successful application of a new inclusion TLC technique with Me-β-CD for the separation of these biologically important bile acids including unconjugated CDCA and DCA and their conjugates.



Unconjugates

	R_1	R_2	R_3	R_4
CA	OH	α-OH	OH	OH
CDCA	OH	α-OH	H	OH
DCA	OH	H	OH	OH
UDCA	OH	β-OH	H	OH
LCA	OH	H	H	OH

Glycine (G) conjugates (R_4= $NHCH_2COOH$)
GCA, GCDCA, GDCA, GUDCA, and GLCA

Taurine (T) conjugates (R_4= $NHCH_2CH_2SO_3Na$)
TCA, TCDCA, TDCA, TUDCA, and TLCA

Sulfates (Sul) (R_1= OSO_3Na, R_4= ONa)
CA 3-Sul, CDCA 3-Sul, DCA 3-Sul, UDCA 3-Sul, and LCA 3-Sul

Glucoside (Glc) conjugates (R_1= G_1, R_4= OH)
CA 3-Glc, CDCA 3-Glc, DCA 3-Glc, UDCA 3-Glc, and LCA 3-Glc

Glucuronide (GlcA) conjugates (R_1= G_2, R_4= OH)
CA 3-GlcA, CDCA 3-GlcA, DCA 3-GlcA, UDCA 3-GlcA, and LCA 3-GlcA

Fig. 1. Structures of unconjugated and conjugated bile acids examined.

The method involves the use of 2D RP–HPTLC technique, developing with aqueous organic solvents in the first dimension and the same solvent system containing Me-β-CD in the second dimension. The structures of bile acids examined in this study are shown in Fig. 1.

2. Experimental

2.1. Materials and reagents

CA, DCA, and LCA were purchased from Wako Pure Chemical Industries, (Osaka, Japan). CDCA and UDCA were kindly donated by Tokyo Tanabe (Tokyo, Japan). The following conjugated bile acids were prepared according to literature methods: amidates with glycine (GCA, GCDCA, GDCA, GUDCA and GLCA) and taurine (as the sodium salts; TCA, TCDCA, TDCA, TUDCA and TCA) [21], 3-sulfates (as the disodium salts; CA 3-Sul, CDCA 3-Sul, DCA 3-Sul, UDCA 3-Sul and LCA 3-Sul) [22], 3-glucosides (CA 3-Glc, CDCA 3-Glc, DCA 3-Glc, UDCA 3-Glc and LCA 3-Glc) [23], and 3-glucuronides (CA 3-GlcA, CDCA 3-GlcA, DCA 3-GlcA, UDCA 3-GlcA and LCA 3-GlcA) [24].

Partition RP–HPTLC plates precoated with C_{18}-bonded silica gel RP-18F_{254S} and adsorption NP–HPTLC plates precoated with silica gel 60F_{254} (each 10×10 cm; particle size, 7 μm; layer thickness, 0.2 mm) were available from Merck (Darmstadt, Germany). Me-β-CD [heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin] was obtained from Sigma Chemical (St. Louis, MO, USA). Sep-Pak plus tC_{18} (sorbent weight, 480 mg) and Sep-Pak SIL (sorbent weight, 690 mg) cartridges for RP- and NP-solid-phase extractions, respectively, were from Waters Associates (Milford, MA, USA); they were preconditioned prior to use. All other reagents and solvents were of analytical reagent or HPLC grades and used without further purification.

2.2. Developing solvent systems

For the separation of individual unconjugated and conjugated bile acids on RP–HPTLC plates, the developing solvent systems used in this study are shown in Table 1. Volumetric compositions represented by symbols A, B, and C in parentheses refer to the developing solvents used for the separation of unconjugated, glycine-amidated, 3-glucosylated and 3-glucuronidated, 3-sulfated, and taurine-amidated bile acids, respectively. For 2D RP–HPTLC, the solvent systems 1A–1C and 2A were used in the first development, and 3A–3C and 4A in the second development. The solvent systems 2A and 4A were recommended by Lepri et al. [14] for the separation of unconjugated bile acids in the 2D RP–HPTLC without Me-β-CD.

2.3. Method

Typical 2D RP–HPTLC with Me-β-CD was performed as follows. A sample solution was prepared by dissolving a bile acid (ca. 0.1 mg) in methanol (100 μl). One μl each of sample solutions was

Table 1
2D RP–HPTLC developing solvents examined

Solvent system	Composition	Direction of development[b]
1	methanol–water[a] (A, 80:20, v/v) (B, 70:30, v/v) (C, 65:35, v/v)	F
2	*n*-hexane–ethyl acetate–acetic acid (A, 72:18:10, v/v/v)	F
3	methanol–water[a] containing 5 m*M* Me-β-CD (A, 80:20, v/v) (B, 70:30, v/v) (C, 65:35, v/v)	S
4	acetic acid–methanol–water (A, 60:20:20, v/v/v)	S

[a] 0.3% sodium phosphate buffer (pH, 7.5).
[b] F and S represent the first and second developments, respectively, in 2D RP–HPTLC.

applied to a RP–HPTLC plate 0.5 cm from the two adjacent edges, using a 1-μl microsyringe. All the developments were carried out in a TLC tank lined with a filter paper at room temperature. The plates were developed with two different appropriate solvent systems in the two different dimensions, until the solvent front migrated 9 cm above the starting point. After the first development with a solvent system (1A–1C or 2A), the plates were removed from the tank, dried with a stream of air and then subjected to the second development with a solvent system (3A–3C or 4A) perpendicular to the first one. The plates were dried, sprayed with 20% phosphomolybdic acid in methanol solution and then heated at 110°C to visualize spots.

2.4. Extraction procedure of glycine-conjugated bile acids from human bile

Glycine-conjugated bile acids in human bile of a patient with cholesterol gallstones were extracted according to the procedures reported previously [25–27]. In short, a bile sample (200 μl) was diluted with 0.5 *M* sodium phosphate buffer (pH 7.0, 4 ml) and passed though a preconditioned Sep-Pak tC_{18} cartridge; after washing with water (5 ml), bile acids were eluted with 90% ethanol (5 ml) and the solvent was evaporated to dryness; the residue was then redissolved in 0.5 *M* sodium phosphate buffer and the above procedure was repeated three times. The bile acid residue was redissolved in ethanol–chloroform–water (20:80:1, v/v/v, 10 ml). The solution was applied to Sep-Pak SIL cartridge at 4°C with elution by gravity flow, and then washed with ethanol–chloroform–water–acetic acid (20:80:1:0.02, v/v/v/v, 2 ml); this fraction contains unconjugated bile acids [26]. Continued elution with ethanol–chloroform–water–acetic acid (25:80:3:5, v/v/v/v, 10 ml) afforded a glycine-conjugated bile acid fraction which was subjected to a 2D RP–HPTLC analysis after concentration of the solvent.

3. Results and discussion

3.1. Preliminary study

Five unconjugated bile acids, CA, CDCA, DCA, UDCA and LCA, and their C-24 glycine and taurine (as sodium salts) conjugates, 3-sulfates (as disodium salts), and 3-glucoside and 3-glucuronide conjugates were examined to study 2D RP–HPTLC separation and behavior (Fig. 1).

Prior to 2D RP–HPTLC analysis, our initial effort was directed to the choice of a suitable TLC plate, developing solvent and a mobile phase additive by using conventional 1D TLC. Based on our previous paper [11], RP–HPTLC plates were employed in this study, because they differ from regular RP–TLC plates in the use of finer particle size adsorbents (5 to 10 μm) which give less diffused spots. Since the formation of stable inclusion complexes of Me–β-CD with solutes proceeds more easily in an aqueous organic solvent [28], methanol was selected as an organic solvent modifier. The use of sodium phosphate buffer as an aqueous modifier, instead of water, was found to depress the tailing of spots because it keeps the mobile phase below the pK_a, particularly with relatively polar taurine and GlcA conjugates of bile acids. Furthermore, Me-β-CD was chosen as a mobile phase additive, as free β-CD is a limited solubility in aqueous organic solvent [29].

3.2. 1D RP–HPTLC behavior of bile acids

On the basis of the preliminary study mentioned above, methanol–water (as 0.3% sodium phosphate buffer; pH, 7.5) mixtures (solvent systems 1A~1C) were chosen as the first eluents and those containing 5 m*M* Me-β-CD (solvent systems 3A~3C) as the second eluents in 2D RP–HPTLC (Table 1). For the purpose of comparison, a combination of nonaqueous hexane–ethyl acetate–acetic acid and aqueous acetic acid–methanol–water mixtures (solvent systems 2A and 4A, respectively) in the two dimensions, proposed by Lepri et al., [14], were also examined.

The R_F values of unconjugated CDCA and DCA and their conjugates on conventional 1D NP- and RP–HPTLC plates are shown in Table 2. Examination of the data revealed that the mobility of the same bile acid differing only in the conjugated forms decreases in the following order on a 1D NP–HPTLC plate, eluting with a chloroform–methanol–acetic acid mixture (24:6:3, v/v/v): unconjugate> glycine - > glucoside - > sulfate - > glucuronide – ≈ taurine-conjugates. On the other hand, these com-

Table 2
R_F values on 1D NP- and RP–HPTLC for unconjugated CDCA and DCA and their conjugates

Mode of conjugation	NP–HPTLC[a]		RP–HPTLC[b]	
	CDCA	DCA	CDCA	DCA
Unconjugate	0.79	0.80	0.17	0.16
Glycine conjugate	0.41	0.40	0.30	0.28
Taurine conjugate	0.11	0.11	0.50	0.47
Sulfate	0.24	0.25	0.46	0.48
Glucoside conjugate	0.35	0.38	0.28	0.27
Glucuronide conjugate	0.11	0.15	0.34	0.34

[a] In NP–HPTLC on silica gel; compounds were developed in chloroform–methanol–acetic acid, 24:6:3 (v/v/v).
[b] In RP–HPTLC on C_{18}-bonded silica gel; compounds were developed in the solvent system 1A.

pounds migrated on a 1D RP–HPTLC plate (solvent system 1A) in the following increasing order: unconjugate<glycine-≈glucoside-<glucuronide-< sulfate-≈taurine-conjugates. The mobilities on RP–TLC are, therefore, not a direct reversal of those found on NP–TLC ones.

The effects of Me-β-CD concentration in eluents on the R_F values of 1D RP–HPTLC were then investigated by using CDCA and DCA as solutes and the eluent systems 3A~3C as developing solvents. As shown in Table 3, the R_F values of CDCA increased sensitively with an increasing concentration of the Me-β-CD, but those of DCA were either barely affected or increased slightly, thus indicating that the former has a larger mobility than the latter under the influence of Me-β-CD. Such phenomena were also observed for all of the analogous CDCA- and DCA-conjugated pairs and suggest that unconjugated CDCA and its conjugates form more stable inclusion complexes with Me-β-CD than the corresponding DCA. The finding in 1D inclusion RP–HPTLC behavior is of special importance in the separation of the two 'difficult' pairs and consistent with that obtained in RP–HPLC with Me-β-CD [29]. In this study, mixtures of methanol–water (as 0.3 m*M* sodium phosphate buffer) containing 5 m*M* Me-β-CD (solvent systems 3A~3C) were employed as the second developing solvents in 2D inclusion RP–HPTLC.

Table 4 shows the R_{F1} and R_{F2} values of thirty bile acids measured on 1D RP–HPTLC plates developed with the solvent systems 1A~1C and 3A~3C, respectively, which differ only in the absence or presence of 5 m*M* Me-β-CD. The table also contains the data for ΔR_F values, which show the differences in the R_{F1} and R_{F2} values for identical compounds.

In conventional 1D RP–HPTLC with the solvent systems 1A~1C, five unconjugated bile acids differing in the number, position and configuration of hydroxyl groups at the positions C-3, C-7 and/or C-12 in the 5β-steroid nucleus were well resolved except for a pair of CDCA and DCA, and the following order of increasing R_{F1} values was observed: LCA<CDCA≈DCA<CA<UDCA. An essentially identical order of the R_{F1} values was observed for individual compounds in each group of unconjugated and conjugated bile acids. The largest R_{F1} values of dihydroxylated UDCA within each homologous series of bile acids can be well explained as the decrease in the hydrophobicity owing to the presence of a 7β-hydroxyl group, which

Table 3
Effect of Me-β-CD concentration on the R_F values of unconjugated CDCA and DCA and their conjugates

Compound	Solvent system	0 m*M*	1 m*M*	3 m*M*	5 m*M*	7 m*M*
CDCA	3A	0.15	0.17	0.20	0.24	0.26
GCDCA	3A	0.28	0.28	0.32	0.36	0.37
TCDCA	3C	0.19	0.20	0.23	0.27	0.29
CDCA 3-Sul	3B	0.22	0.26	0.31	0.35	0.38
CDCA 3-Glc	3A	0.27	0.31	0.33	0.36	0.38
CDCA3-GlcA	3A	0.36	0.38	0.41	0.46	0.46
DCA	3A	0.15	0.14	0.15	0.16	0.16
GDCA	3A	0.28	0.28	0.27	0.29	0.29
TDCA	3C	0.17	0.17	0.16	0.17	0.19
DCA3-Sul	3B	0.21	0.24	0.24	0.26	0.26
DCA3-Glc	3A	0.26	0.25	0.26	0.26	0.28
DCA3-GlcA	3A	0.33	0.33	0.35	0.37	0.37

Table 4
R_F data for unconjugated and conjugated bile acids on 1D RP–HPTLC

Compound	R_{F1}^{a}	R_{F2}^{b}	ΔR_F^{c}
Unconjugate	(1A)[d]	(3A)[d]	
CA	0.29	0.34	0.05
CDCA	0.17	0.28	0.11
DCA	0.16	0.19	0.03
UDCA	0.34	0.54	0.20
LCA	0.09	0.23	0.14
Glycine conjugate	(1A)	(3A)	
GCA	0.46	0.50	0.04
GCDCA	0.30	0.39	0.09
GDCA	0.30	0.33	0.03
GUDCA	0.54	0.62	0.08
GLCA	0.17	0.30	0.13
Taurine conjugate	(1C)	(3C)	
TCA	0.33	0.38	0.05
TCDCA	0.22	0.31	0.09
TDCA	0.18	0.20	0.02
TUDCA	0.44	0.53	0.09
TLCA	0.12	0.27	0.15
Sulfate	(1B)	(3B)	
CA 3-Sul	0.31	0.37	0.06
CDCA 3-Sul	0.20	0.37	0.17
DCA 3-Sul	0.20	0.27	0.07
UDCA 3-Sul	0.39	0.56	0.17
LCA 3-Sul	0.13	0.34	0.21
Glucoside conjugate	(1A)	(3A)	
CA 3-Glc	0.40	0.43	0.03
CDCA 3-Glc	0.28	0.37	0.09
DCA 3-Glc	0.26	0.28	0.02
DCA 3-Glc	0.54	0.61	0.07
LCA 3-Glc	0.17	0.34	0.17
Glucuronide conjugate	(1A)	(3A)	
CA3-GlcA	0.51	0.54	0.03
CDCA 3-GlcA	0.37	0.49	0.12
DCA 3-GIcA	0.37	0.41	0.04
UDCA3-GIcA	0.61	0.71	0.10
LCA 3-GlcA	0.27	0.47	0.20

[a] R_F values obtained in the solvent systems without Me-β-CD.
[b] R_F values obtained in the solvent systems with Me-β-CD.
[c] Differences in the R_{F2} and R_{F1} values for corresponding compounds.
[d] Symbols in parentheses refer to solvent systems used (see Table 1).

reduces its affinity with a C_{18}-bonded stationary phase [30]. In analogy with previous papers [4–6], all of the isomeric pairs of unconjugated CDCA and DCA and their conjugates had similar R_{F1} values on 1D RP–HPTLC plates.

In 1D inclusion RP–HPTLC with the solvent systems 3A~3C, addition of 5 m*M* Me-β-CD in the developing solvents resulted in the increases of the mobility ($R_{F2} > R_{F1}$) for all the compounds examined, and the magnitude of the increases (ΔR_F value) depend exclusively on the structure of the solutes. Thus the ΔR_F values of CDCA, UDCA and LCA in each group are always much larger than those of CA and DCA, which have a 12α-hydroxyl group in the 5β-steroid nucleus. As the increments of the ΔR_F values are almost independent of the other structural characteristics such as the modes of conjugation, the determination of the ΔR_F value for an unknown bile acid affords a useful indication not only for estimating the presence or absence of a 12α-hydroxyl group, but also for differentiating CDCA and DCA in each homologous series of both the unconjugate and conjugate pairs.

As a result, the R_{F2} values of individual compounds in each homologous series on 1D inclusion RP–HPTLC with Me-β-CD in aqueous organic mobile phases differed from the R_{F1} values observed in the corresponding conventional 1D RP–HPTLC without Me-β-CD, and the following order of increasing R_{F2} valves was observed for unconjugated bile acids: DCA<LCA<CDCA<CA<UDCA. The corresponding conjugated bile acids within each homologous series also followed essentially a similar R_{F2} order except for LCA. The inclusion RP–HPTLC with Me-β-CD make the separation of the recalcitrant pairs of unconjugated CDCA and DCA and their conjugates possible, which cannot be separated easily on previously reported TLC techniques. However, a problem arose anew from the 1D inclusion RP–HPTLC which was the interference from the spots of LCA and its conjugates, which lie close to those of the corresponding CDCA and DCA. Therefore, a more adequate TLC condition for completely separating five bile acids in each homologous series was undertaken.

3.3. 2D RP–HPTLC behavior of bile acids

We have previously reported the effectiveness of 1D RP–HPTLC for the separation of the di- and trihydroxy stereoisomers of unconjugated bile acids [11]. Subsequently, Lepri, et al., [14] applied RP–HPTLC plates to the 2D technique by developing

with nonaqueous and aqueous organic mobile phases in two different directions and succeeded in the separation of unconjugated CDCA and DCA. Meanwhile, a number of the positional, geometrical, and optical isomers of bioactive compounds other than bile acids have been successfully resolved by inclusion HPLC with a β-CD [20,29]. However, no TLC method has been able to separate distinctly five major bile acids in both the unconjugated and conjugated forms.

On the basis of the R_{F1} and R_{F2} data for individual compounds obtained from the conventional and 1D inclusion RP–HPTLCs mentioned above, the theoretical 2D chromatograms were simulated. The typical 2D simulations of some groups of bile acids are shown in Fig. 2(A), in which five major bile acids in each homologous series were completely resolved without interference of LCA or its conjugates. On the other hand, actual 2D chromatograms measured for the corresponding bile acids are shown in Fig. 2(B). As expected, the experimental 2D chromatograms were found to be very similar to the corresponding theoretical ones. The unconjugated bile acids and their glycinated, 3-glucosylated, and 3-glucuronidated conjugates were developed in the first dimension with the solvent system 1A and in the second dimension with the solvent system 3A. More polar taurine-conjugated (or 3-sulfated) bile acids were developed with the solvent systems 1C (or 1B) and 3C (or 3B) in the two different directions. Thus all of the six 'difficult-to-separate' pairs, CDCA vs. DCA, GCDCA vs. GDCA, TCDCA vs. TDCA, CDCA 3-Sul vs. DCA 3-Sul, CDCA 3-Glc vs. DCA 3-Glc and CDCA 3-GlcA vs. DCA 3-GlcA, were nicely resolved by the 2D inclusion RP–HPTLC technique with Me-β-CD.

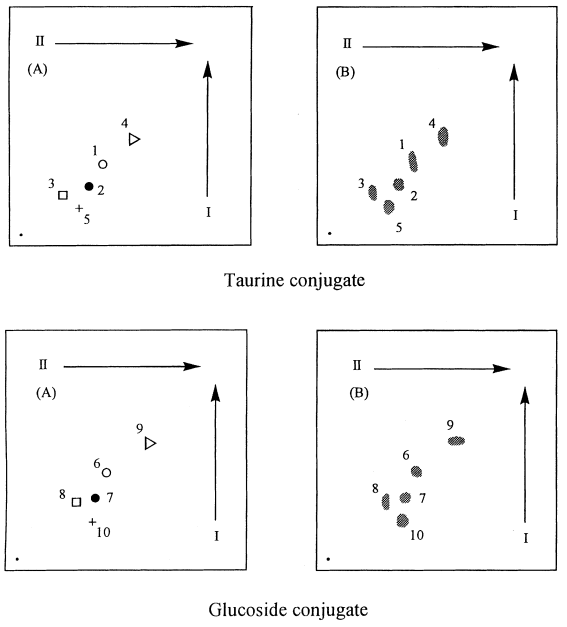


Fig. 2. Theoretical (A) and experimental (B) 2D inclusion RP–HPTLC with Me-β-CD of a mixture of the taurine- and glucoside-conjugated bile acids. I and II represent the direction of the first and second developments, respectively. Taurine conjugates: developing solvent, 1C and 3C in the first and second directions, respectively; spot identification, 1=TCA, 2=TCDCA, 3=TDCA, 4=TUDCA, 5=TLCA. Glucoside conjugates: developing solvent, 1A and 3A in the first and second directions, respectively; spot identification, 6=CA 3-Glc, 7=CDCA 3-Glc, 8=DCA 3-Glc, 9=UDCA 3-Glc, 10=LCA 3-Glc.

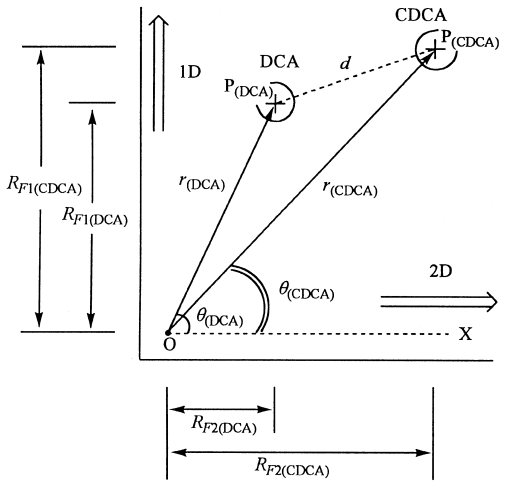
Table 5 shows the theoretical and experimental 2D inclusion RP–HPTLC data for unconjugated CDCA and DCA and their conjugates. The theoretical data were obtained from the R_{F1} and R_{F2} values shown in Table 1. The position of each spot in the 2D chromatograms was expressed as the polar coordinates (r, θ), where r and θ are the distance from the origin and the angle measured from the x-axis, respectively (Fig. 3). Also, the degree of separation of two corresponding compounds was represented by the distance (d) between the two spots, which were calculated from the r and θ values of the same compound. As can be seen in the Table 5, the experimental (r, θ) values for CDCA and DCA in each pair appreciably differed from each other, and all the d values were more than 0.06, when they were measured by the combined use of the solvent systems 1A~1C and 3A~3C. In addition, the experimental d values in each pair agreed very well with the theoretical ones.

For the separation of unconjugated CDCA and DCA, a combination of nonaqueous and aqueous mobile phases (solvent systems 2A and 3A, respectively) in the two different dimensions provided a more satisfactory result (d=0.18). However, the use of the solvent system 2A in the first direction is unsuitable for the separation of more polar conjugated bile acids. Analogously, a much more excellent separation of CDCA and DCA was attained by the use of the solvent systems 1A and 3A than that of

Table 5
2D inclusion RP–HPTLC data for unconjugated CDCA and DCA and their conjugates

Mode of conjugation	Solvent system (F, S)[e]	Theoretical value[a]			Experimental value[b]		
		Position of spot[c]		d[d]	Position of spot[c]		d[d]
		CDCA (r, θ)	DCA (r, θ)		CDCA (r, θ)	DCA (r, θ)	
Unconjugate	(1A, 3A)	(0.33, 31)	(0.25, 40)	0.09	(0.30, 37)	(0.22, 46)	0.09
	(2A, 3A)				(0.67, 64)	(0.52, 73)	0.18
	(2A, 4A)				(0.70, 61)	(0.73, 64)	0.05
Glycine conjugate	(1A, 3A)	(0.49, 39)	(0.45, 42)	0.04	(0.51, 42)	(0.45, 42)	0.06
Taurine conjugate	(1C, 3C)	(0.38, 35)	(0.30, 37)	0.12	(0.30, 42)	(0.22, 47)	0.08
Sulfate	(1B, 3B)	(0.42, 29)	(0.33, 36)	0.10	(0.44, 35)	(0.35, 43)	0.11
Glucoside conjugate	(1A, 3A)	(0.45, 38)	(0.38, 43)	0.08	(0.54, 35)	(0.45, 42)	0.11
Glucuronide conjugate	(1A, 3A)	(0.61, 37)	(0.55, 42)	0.08	(0.56, 49)	(0.48, 54)	0.09

[a] Data obtained indirectly from 1D chromatograms.
[b] Data obtained directly from 2D chromatograms.
[c] Polar coordinates (see Fig. 3).
[d] Distance between the two spots (see Fig. 3).
[e] Symbols in parentheses refer to solvent systems used (see Table 1).



$$\theta_{(DCA)} = \angle P_{(DCA)}\,OX$$
$$\theta_{(CDCA)} = \angle P_{(CDCA)}\,OX$$
$$r_{(DCA)} = [R_{F2(DCA)}^2 + R_{F1(DCA)}^2]^{1/2}$$
$$r_{(CDCA)} = [R_{F2(CDCA)}^2 + R_{F1(CDCA)}^2]^{1/2}$$
$$d = \{r_{(DCA)}^2 + r_{(CDCA)}^2 - 2r_{(DCA)}\,r_{(CDCA)}\cos[\theta_{(DCA)} - \theta_{(CDCA)}]\}^{1/2}$$

Fig. 3. Definition of polar coordinates of a spot and distance between two different spots on 2D RP–HPTLC.

2A and 4A (d=0.05) recommended by Lepri, et al., [14]. Thus aqueous methanol without and with Me-β-CD as the developing solvents in 2D RP–HPTLC are also superior to other solvent systems from the standpoint of wide applicability and simplicity to use.

3.4. Application of the method to human biological materials

In order to evaluate the applicability of the present method, we carried out the separation of glycine-conjugated bile acids extracted from the gall bladder bile of a patient with cholesterol gallstones. A typical 2D chromatogram is shown in Fig. 4. Each spot was cleanly separated from other spots and readily identified by comparison of the chromatographic behavior with that of the authentic samples. GCA, GCDCA, GDCA, and GUDCA were detected as main glycine-conjugated bile acids in this bile specimen, in accord with the result of a CGC analysis reported previously by us [31].

In conclusion, the 2D inclusion RP–HPTLC method with Me-β-CD provides an excellent separation of individual bile acids, particularly of CDCA and DCA in both the unconjugated and conjugated forms, developing with aqueous methanol in the first direction and the same solvent system containing

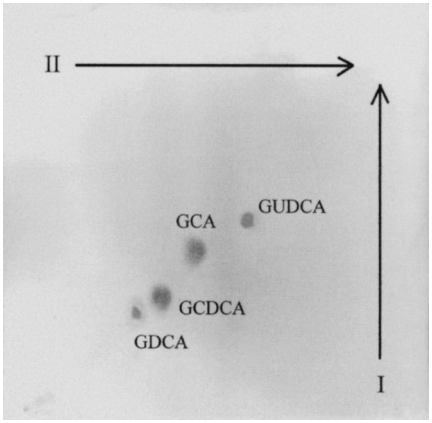


Fig. 4. 2D inclusion RP–HPTLC with Me-β-CD of glycine-conjugated bile acids extracted from human gallbladder bile. Developing solvent, 1A and 3A in the first and second directions, respectively.

Me-β-CD in the second direction. The proposed method, because of the simplicity and efficiency, should be applied to the preliminary and routine analyses of a mixture of these biologically important bile acids present in human and animal biological materials.

4. List of abbreviations

TLC	thin-layer chromatography
HPTLC	high-performance thin-layer chromatography
Me-β-CD	methyl β-cyclodextrin
2D	two-dimensional
NP	normal-phase
RP	reversed-phase
HPLC	high-performance liquid chromatography
CGC	capillary gas chromatography
CA	cholic (3α,7α,12α-trihydroxy-5β-cholan-24-oic) acid
CDCA	chenodeoxycholic (3α,7α-dihydroxy-5β-cholan-24-oic) acid
DCA	deoxycholic (3α,12α-dihydroxy-5β-cholan-24-oic) acid;
UDCA	ursodeoxycholic(3α,7β-dihydroxy-5β-cholan-24-oic) acid
LCA	lithocholic (3α-hydroxy-5β-cholan-24-oic) acid
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
GUDCA	glycoursodeoxycholic acid
GLA	glycolithocholic acid
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TUDCA	tauroursodeoxycholic acid
TLCA	taurolithocholic acid
CA 3-Sul	cholic acid 3-sulfate
CDCA 3-Sul	chenodeoxycholic acid 3-sulfate
DCA 3-Sul	deoxycholic acid 3-sulfate
UDCA 3-Sul	ursodeoxycholic acid 3-sulfate
LCA 3-Sul	lithocholic acid 3-sulfate
CA 3-Glc	cholic acid 3-glucoside
CDCA 3-Glc	chenodeoxycholic acid 3-glucoside
DCA 3-Glc	deoxycholic acid 3-glucoside
UDCA 3-Glc	ursodeoxycholic acid 3-glucoside
LCA 3-Glc	lithocholic acid 3-glucoside
CA 3-GlcA	cholic acid 3-glucuronide
CDCA 3-GlcA	chenodeoxycholic acid 3-glucuronide
DCA 3-GlcA	deoxycholic acid 3-glucuronide
UDCA 3-GlcA	ursodeoxycholic acid 3-glucuronide
LCA 3-GlcA	lithocholic acid 3-glucuronide.

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