Journal of Chromatography, 276 (1983) 289—300
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1751

STUDIES ON STEROIDS

CLXXXVII. DETERMINATION OF SERUM BILE ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE LABELING

JUNICHI GOTO, MAKOTO SAITO, TOSHIYUKI CHIKAI, NOBUHARU GOTO and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Sendai 980 (Japan)

(Received February 16th, 1983)

SUMMARY

A method for the simultaneous determination of bile acids in serum by high-performance liquid chromatography (HPLC) with fluorescence labeling is described. The bile acid fraction was obtained from a serum specimen by passing it through a BondElut cartridge. Bile acids were derivatized quantitatively into the fluorescent compounds through the hydroxyl group at C-3 by treatment with 1-anthroyl nitrile in the presence of quinuclidine in acetonitrile. These derivatives were separated into the free, glycine- and taurine-conjugate fractions by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Subsequent resolution of each fraction into cholate, ursodeoxycholate, chenodeoxycholate, deoxycholate and lithocholate was attained by HPLC on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer (pH 6.0)—methanol (1:5) and 0.1% potassium phosphate buffer (pH 6.0)—methanol (1:8) as mobile phases. The anthroyl bile acids were monitored by fluorescence detection (excitation wavelength 370 nm; emission wavelength 470 nm), the limit of detection being 20 fmol. The proposed method proved to be applicable to the quantitation of bile acids in serum with satisfactory reliability and sensitivity.

INTRODUCTION

In recent years considerable attention has been directed to the biodynamics of bile acids in patients with hepatobiliary diseases. For this purpose the development of a reliable method for the profile analysis of bile acids in biological fluids is urgently needed. Among various methods high-performance liquid chromatography (HPLC) with fluorescence detection appears to be most

0378-4347/83/$03.00

promising with respect to resolution, sensitivity and versatility. Pre-column labeling with a fluorophore usually involves the carboxyl group of the side-chain [1, 2]. This method, however, requires hydrolysis of the taurine conjugate prior to fluorescence labeling. Recently, novel methods using immobilized 3α-hydroxysteroid dehydrogenase have been developed for the determination of bile acids in serum [3—5]. These procedures, however, have disadvantages in that the resolution of chenodeoxycholate and deoxycholate is unsatisfactory and the sensitivity is still insufficient. In the previous study we developed a new type of fluorescence labeling reagent having a carbonyl nitrile group for use in HPLC of the hydroxyl compounds [6, 7]. The present paper deals with the use of 1-anthroyl nitrile for the derivatization of serum bile acids through the 3α-hydroxyl group, followed by separation and determination by HPLC with fluorescence detection.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus was a Model 638-50 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model 650-10LC fluorescence spectrophotometer (Hitachi) (excitation wavelength 370 nm; emission wavelength 470 nm). A Cosmosil $5C_{18}$ (5 μm) column (15 cm × 4 mm I.D.) (Nakarai Kagaku Co., Kyoto, Japan) was used at ambient temperature.

Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid were purchased from Sigma (St. Louis, MO, U.S.A.) and purified prior to use. Ursodeoxycholic acid was kindly donated by Tokyo Tanabe Co. (Tokyo, Japan). The glycine and taurine conjugates were synthesized by the *p*-nitrophenyl ester method in these laboratories [8, 9]. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All the reagents employed were of analytical grade. Solvents were purified by distillation prior to use. Piperidinohydroxypropyl Sephadex LH-20 (acetate form, PHP-LH-20) [10] (0.6 mequiv./g) and 1-anthroyl nitrile [7] were prepared in the manner described in the previous papers. A BondElut cartridge (Analytichem International, Harbor City, CA, U.S.A.) was washed successively with ethanol (5 ml) and water (5 ml) prior to use. All glassware used was silanized with trimethylchlorosilane.

Preparation of free and conjugated deoxycholate 12-propionates

Deoxycholic acid 12-propionate. To a solution of methyl deoxycholate (2 g) in pyridine (20 ml) was added propionyl chloride (4 ml). The resulting solution was heated at 60°C for 50 min and then poured into ice—water. After extraction with ethyl acetate, the organic layer was washed successively with 5% $NaHCO_3$, water, 3% hydrochloric acid and water, and evaporated down. An oily residue obtained was dissolved in 3% methanolic potassium hydroxide (15 ml) and allowed to stand at room temperature for 4 h. After neutralization with concentrated hydrochloric acid, the solution was concentrated in vacuo, poured into ice-cooled 1% potassium hydroxide and

extracted with diethyl ether. The aqueous layer was acidified with concentrated hydrochloric acid, and the precipitate was collected by filtration and washed with water. Recrystallizaton from acetone gave deoxycholic acid 12-propionate (1.5 g) as colorless needles, m.p. 214—215°C. Anal. calc. for $C_{27}H_{44}O_5$: C, 72.28; H, 9.89. Found C, 72.43; H, 10.01. NMR (C^2HCl_3) δ: 0.72 (3H, s, 18-CH_3), 0.81 (3H, d, J = 6 Hz, 21-CH_3), 0.88 (3H, s, 19-CH_3), 1.17 (3H, t, J = 8 Hz, CH_3CH_2—), 2.35 (2H, q, J = 8 Hz, CH_3CH_2—), 3.62 (1H, m, 3β-H), 5.06 (1H, m, 12β-H).

Glycodeoxycholic acid 12-propionate. To a solution of deoxycholic acid 12-propionate (1 g) in ethyl acetate (40 ml) were added N-hydroxysuccinimide (700 mg) and N,N'-dicyclohexylcarbodiimide (1.5 g), and the solution was stirred at room temperature overnight. The precipitate was removed by filtration and the filtrate was washed with water. After evaporation of the solvent, an oily residue obtained was subjected to column chromatography on silica gel (30 g). Elution with hexane—ethyl acetate and recrystallization of the eluate from hexane—acetone gave deoxycholate N-succinimidyl ester 12-propionate (800 mg) as colorless needles, m.p. 164—165°C. NMR (C^2HCl_3) δ: 0.75 (3H, s, 18-CH_3), 0.85 (3H, d, J = 6 Hz, 21-CH_3), 0.90 (3H, s, 19-CH_3), 1.18 (3H, t, J = 8 Hz, CH_3CH_2—), 2.37 (2H, q, J = 8 Hz, CH_3CH_2—), 2.80 (4H, s, —$COCH_2CH_2CO$—), 3.60 (1H, m, 3β-H), 5.03 (1H, m, 12β-H). To a solution of deoxycholate N-succinimidyl ester 12-propionate (700 mg) in ethyl acetate (15 ml) was added ethyl glycinate · HCl (800 mg) in pyridine (3 ml), and the resulting solution was heated at 60°C for 48 h. The reaction mixture was poured into ice—water and extracted with ethyl acetate. The organic layer was washed with 5% hydrochloric acid and water, and evaporated down. An oily residue obtained was subjected to column chromatography on silica gel (25 g) with hexane—ethyl acetate. Ethyl glycodeoxycholate 12-propionate (200 mg) thus obtained was dissolved in 1.5% methanolic potassium hydroxide (10 ml) and allowed to stand at room temperature for 1 h. The reaction mixture was neutralized with concentrated hydrochloric acid, concentrated in vacuo, added with 3% hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with water and evaporated down. An oily residue was subjected to column chromatography on silica gel (6 g). Elution with chloroform—methanol and recrystallization of the eluate from acetonitrile—methanol gave glycodeoxycholate 12-propionate (100 mg) as a colorless crystalline substance, m.p. 178—180°C. Anal. calc. for $C_{29}H_{47}NO_6$: C, 68.88; H, 9.37; N, 2.77. Found: C, 68.61; H, 9.07; N, 2.87. NMR ($C^2H_3O^2H$) δ: 0.78 (3H, s, 18-CH_3), 0.87 (3H, d, J = 6 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 1.17 (3H, t, J = 8 Hz, CH_3CH_2—), 2.41 (2H, q, J = 8 Hz, CH_3CH_2—), 3.54 (1H, m, 3β-H), 3.83 (2H, s, >NCH_2—), 5.08 (1H, m, 12β-H).

Taurodeoxycholate 12-propionate. To a solution of deoxycholate N-succinimidyl ester 12-propionate (200 mg) in pyridine (5 ml) was added taurine (600 mg) in water (1 ml), and the resulting solution was allowed to stand at room temperature overnight. After evaporation of the solvent, the oily residue obtained was subjected to column chromatography on silica gel (8 g). Elution with chloroform—methanol and recrystallization of the eluate from aceto-

nitrile—methanol gave taurodeoxycholate 12-propionate (70 mg) as a colorless crystalline substance, m.p. 162—165°C. Anal. calc. for $C_{29}H_{49}NO_7S \cdot H_2O$: C, 60.70; H, 8.96; N, 2.44. Found: C, 60.51; H, 9.12; N, 2.51. NMR ($C^2H_3O^2H$) δ: 0.77 (3H, s, 18-CH_3), 0.85 (3H, d, J = 6 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 1.18 (3H, t, J = 8 Hz, CH_3CH_2—), 2.41 (2H, q, J = 8 Hz, CH_3CH_2—), 2.96 (2H, t, J = 7 Hz, >NCH_2CH_2—), 3.52 (1H, m, 3β-H), 3.59 (2H, t, J = 7 Hz, >NCH_2CH_2—), 5.08 (1H, m, 12β-H).

Procedure for determination of serum bile acids

To a serum sample (100 μl) were added free, glycine- and taurine-conjugated deoxycholate 12-propionates (each 250 ng) as internal standards (I.S.), and the mixture was diluted with 0.5 *M* phosphate buffer (pH 7.0) (1 ml) and applied to a BondElut cartridge. After successive washing with water (2 ml) and 1.5% ethanol (1 ml), bile acids were eluted with 90% ethanol (2 ml). A 400-μl aliquot of the effluent was evaporated down, added with 1-anthroyl nitrile (200 μg) in acetonitrile (100 μl) and 0.16% quinuclidine in acetonrile (100 μl), and the mixture was heated at 60°C for 20 min. After addition of methanol (50 μl) for decomposing excess 1-anthroyl nitrile, the mixture was evaporated down under nitrogen. The residue was dissolved in 90% ethanol (1 ml) and applied to a PHP-LH-20 column (100 mg, 18 mm × 6 mm I.D.). Elution was carried out at a flow-rate of 0.2 ml/min. After washing with 90% ethanol (1 ml), free, glycine- and taurine-conjugated bile acids were fractionally separated by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (5 ml), 0.2 *M* formic acid in 90% ethanol (5 ml), and 0.3 *M* acetic acid—potassium acetate (pH 6.3) in 90% ethanol (5 ml). Each fraction was evaporated and the residue obtained was redissolved in methanol (100—200 μl). A 5—10 μl aliquot of the solution was injected into the HPLC system.

When an interfering peak appeared on a chromatogram of the taurine-conjugate fraction, the following prior clean-up was recommended (see Fig. 6): the eluate from a BondElut cartridge was applied to a PHP-LH-20 column (100 mg, 18 mm × 6 mm I.D.) and eluted with 0.3 *M* acetic acid—potassium acetate (pH 6.3) in 90% ethanol (5 ml). The effluent was evaporated down and then applied to a BondElut cartridge in the manner described above for the elimination of inorganic salts.

Recovery test for bile acids

The test samples were prepared by dissolving 200 pmol each of free, glycine- and taurine-conjugated bile acids in human serum (100 μl). After addition of internal standard (each 250 ng), the serum sample was subjected successively to clean-up by a BondElut cartridge, derivatization with 1-anthroyl nitrile, group separation on PHP-LH-20 and determination by HPLC in the manner described above.

RESULTS AND DISCUSSION

Derivatization of bile acids with 1-anthroyl nitrile

In the previous study we developed 1-anthroyl nitrile as a fluorescence

labeling reagent which is effective for a secondary hydroxyl group on the steroid nucleus [7]. Initially, suitable conditions were investigated for the coupling of bile acids through the inherent 3α-hydroxyl group with 1-anthroyl nitrile (Fig. 1). Bile acids were dissolved in various concentrations of triethylamine or quinuclidine in acetonitrile and allowed to stand at 60° C. An aliquot of the resulting solution was applied to HPLC. The yield of the anthroyl derivative was calculated by comparison with the peak area of the standard sample. The reaction rate was significantly influenced by the organic base as illustrated in Fig. 2. In the presence of triethylamine, the yield of the anthroyl derivative was approximately 40% at 1 h. On the other hand, when quinuclidine, a sterically rigid base, was employed, the reaction rate increased along with the reaction time up to 10 min, resulting in the quantitative formation of the anthroyl derivative. In this condition the 7α and 12α axial and 7β equatorial hydroxyl groups underwent no reaction with 1-anthroyl nitrile due to the steric hindrance. Consequently, derivatization occurred selectively at the 3α equatorial hydroxyl group. On the basis of these data, bile acids were treated with 1-anthroyl nitrile in 0.08% quinuclidine in acetonitrile at 60° C for 20 min.

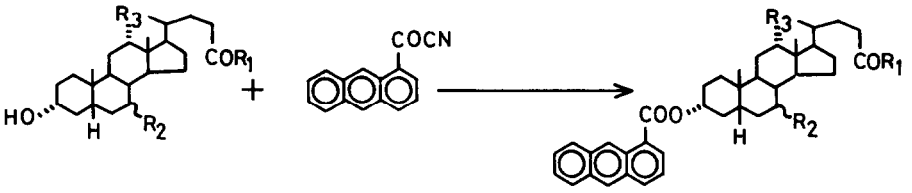

Fig. 1. Transformation of bile acids with 1-anthroyl nitrile into the 3-(1-anthroyl) derivatives. R_1 = OH, $NHCH_2COOH$, $NHCH_2CH_2SO_3H$; R_2 = H, α-OH, β-OH; R_3 = H, α-OH.

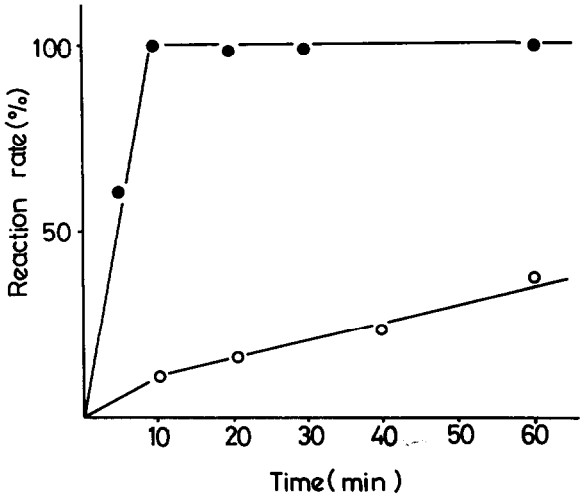

Fig. 2. Time course for derivatization of bile acids with 1-anthroyl nitrile. (•), 0.08% quinuclidine in acetonitrile; (○), 4% triethylamine in acetonitrile.

Separation of bile acid 3-(1-anthroyl) derivatives

Next, effort was directed to the separation of the derivatized bile acids by means of HPLC. Several attempts have been made on the separation of bile acids on ODS columns with various solvent systems [11—15]. In acidic conditions, distinct differences in the k' value are observed among the conjugated forms, but chenodeoxycholate and deoxycholate are not completely resolved. On the other hand, when neutral or weakly alkaline mobile phase is used, the steric interaction between the 12α-hydroxyl group and acidic group of the side-chain takes place, providing efficient separation of the two bile acids [16]. Accordingly, the mobile phase adjusted to pH 6.0—7.8 was employed in the present study.

Various combinations of buffer solution and organic solvent were examined for the suitable mobile phase on Cosmosil $5C_{18}$, which is an ODS column. The use of a potassium phosphate buffer—methanol system appeared to be promising, as it exerts no significant leading or tailing. The chromatographic behaviours of bile acids were investigated in these conditions. The k' values of free bile acids relative to ursodeoxycholic acid were plotted against the pH value using 0.3% potassium phosphate buffer—methanol (1:5) as mobile phase (Fig. 3). Similar chromatographic behaviours were also observed for the glycine and taurine conjugates. It is to be noted that ursodeoxycholate showed a larger k' value than cholate. Although the resolution value of chenodeoxycholic acid and deoxycholic acid decreased along with decreasing pH, the baseline separa-

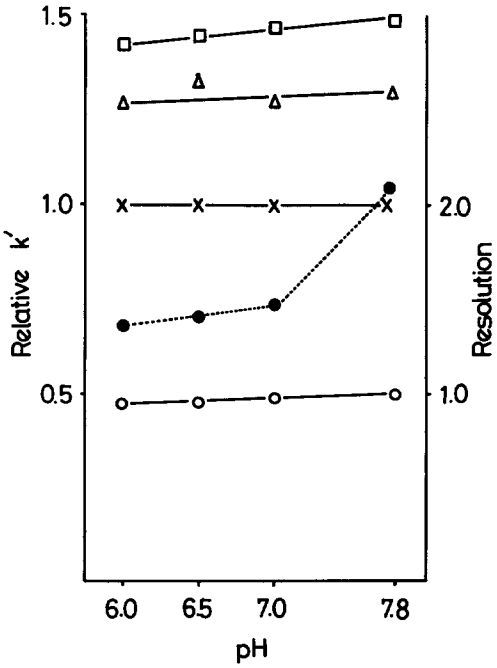

Fig. 3. Effect of pH on the k' values relative to ursodeoxycholate. (○), cholate; (×), ursodeoxycholate; (△), chenodeoxycholate; (□), deoxycholate. The dotted line represents the resolution of chenodeoxycholate and deoxycholate.

tion of these two was achieved even at pH 6.0. The effect of salt concentration in the mobile phase on the retention value was also examined with potassium phosphate buffer (pH 6.0)—methanol (1:5). The k' value was raised with increasing salt concentration up to 0.5%.

On the basis of these data 0.3% potassium phosphate buffer (pH 6.0)—methanol (1:5) and 0.1% potassium phosphate buffer (pH 6.0)—methanol (1:8) were chosen as suitable mobile phases. The k' values of bile acids observed with the two solvent systems are listed in Table I. Typical chromatograms of standard samples are shown in Fig. 4. The anthroyl bile acids were monitored by fluorescence detection (excitation wavelength 370 nm; emission wavelength 470 nm), the limit of detection being 20 fmol. A calibration graph was constructed by plotting the ratio of the peak area of each bile acid to that of free, glycine-, or taurine-conjugated deoxycholic acid 12-propionate against the amount of bile acid.

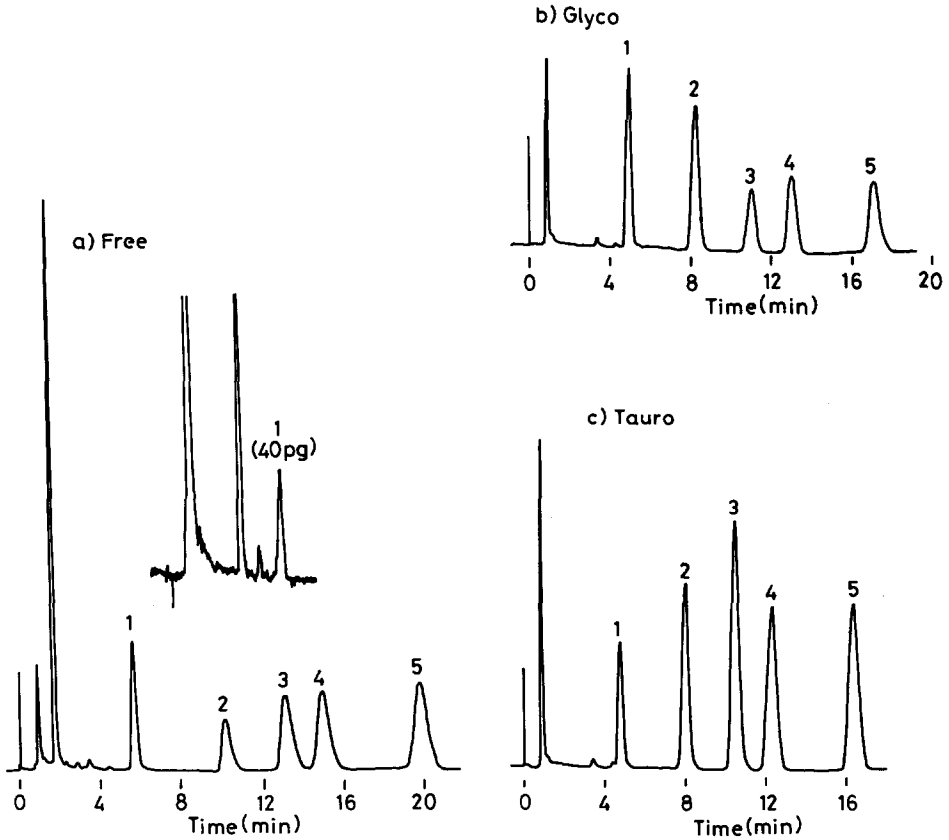

Fig. 4. Separation of 3-(1-anthroyl) derivatives of free and conjugated bile acids. Peaks: 1, cholate; 2, ursodeoxycholate; 3, chenodeoxycholate; 4, deoxycholate; 5, deoxycholate 12-propionate (I.S.).

TABLE I

CAPACITY RATIOS OF 3-(1-ANTHROYL) DERIVATIVES OF FREE AND CONJUGATED BILE ACIDS

Conditions: column, Cosmosil $5C_{18}$; mobile phases, (A) 0.3% potassium phosphate buffer (pH 6.0)—methanol (1:5), (B) 0.1% potassium phosphate buffer (pH 6.0)—methanol (1:8), t_0 = 0.7 min, flow-rate 1.8 ml/min.

Compound	Free		Glycine conjugate (G)		Taurine conjugate (T)	
	A	B	A	B	A	B
Cholate (CA)	7.7	—	7.1	—	6.7	—
Ursodeoxycholate (UDCA)	15.7	—	14.0	—	12.7	—
Chenodeoxycholate (CDCA)	20.5	—	17.5	—	16.8	—
Deoxycholate (DCA)	23.6	5.2	20.7	—	19.9	—
Lithocholate (LCA)	—	15.0	—	14.9	—	14.7
Deoxycholate 12-propionate (I.S.)	30.9	7.7	29.0	—	24.4	—

Clean-up of serum bile acids

The determination of bile acids in blood is markedly influenced by the clean-up procedure employed. For this purpose Amberlite XAD-2 resin has been widely used. This method, however, is not always satisfactory with respect to the recovery rate and reproducibility. In the present study a BondElut cartridge was employed for the clean-up of bile acids in serum. A synthetic mixture of 400 pmol of each of the bile acids was dissolved in 0.5 *M* phosphate buffer (pH 7.0) and applied to the cartridge impregnated with 0.5 *M* phosphate buffer (pH 7.0). After elimination of co-existing substances by washing with water and 1.5% ethanol, bile acids were eluted with 90% ethanol and then determined by HPLC. Bile acids were recovered at a rate of more than 90% in an initial 1.5 ml of the effluent.

It is evident from the data in Table I that each bile acid exhibited an almost identical k' value irrespective of the structure of the side-chain. Therefore, the group separation of bile acids into the three conjugated forms became a prerequisite. A synthetic mixture of anthroyl bile acids was dissolved in 90% ethanol and applied to a column of PHP-LH-20 (acetate form), which is a lipophilic ion-exchange gel. After removal of neutral compounds by elution with 90% ethanol, the group separation was carried out by stepwise elution with 0.1 *M* acetic acid in 90% ethanol, 0.2 *M* formic acid in 90% ethanol and 0.3 *M* acetic acid–potassium acetate (pH 6.3) in 90% ethanol. As shown in Fig. 5, free, glycine- and taurine-conjugated bile acids were completely separated into the three groups.

Determination of bile acids in human serum

A standard procedure for the separation and determination of bile acids in human serum is shown in Fig. 6. As for some serum specimens, especially the pooled human serum, an interfering peak appeared on a chromatogram of the

taurine-conjugate fraction. This problem was readily overcome by ion-exchange chromatography on PHP-LH-20 prior to dervatization with 1-anthroyl nitrile.

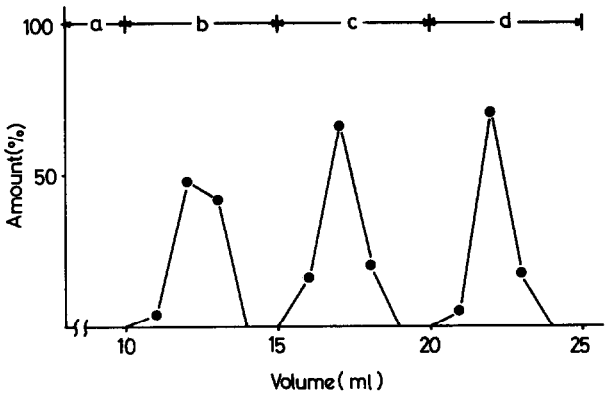

Fig. 5. Group separation of 3-(1-anthroyl) derivatives of bile acids on PHP-LH-20. Eluent: a, 90% ethanol; b, 0.1 *M* acetic acid in 90% ethanol; c, 0.2 *M* formic acid in 90% ethanol; d, 0.3 *M* acetic acid–potassium acetate (pH 6.3) in 90% ethanol.

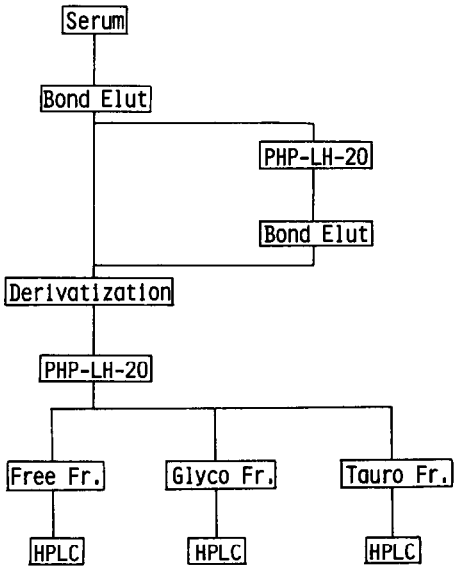

Fig. 6. General scheme for separation and determination of bile acids in human serum.

Applying the standard procedure to human serum, bile acids were determined with satisfactory reproducibility. Known amounts of bile acids were added to human serum and their recovery rates were estimated. As listed in Table II, all bile acids were recovered at a rate of more than 90%. A typical

TABLE II

RECOVERY OF FREE AND CONJUGATED BILE ACIDS ADDED TO NORMAL HUMAN SERUM

Bile acid*	Serum	Added (nmol per 0.1 ml)	Expected (nmol per 0.1 ml)	Found (nmol per 0.1 ml)	Recovery ± S.D.** (%)
CA	<0.005	0.20	0.20	0.196	98.2 ± 4.8
UDCA	0.02	0.20	0.22	0.219	99.6 ± 5.9
CDCA	0.07	0.20	0.27	0.272	100.9 ± 3.6
DCA	0.05	0.20	0.25	0.249	99.4 ± 3.8
LCA	<0.005	0.20	0.20	0.198	99.2 ± 7.5
GCA	<0.005	0.20	0.20	0.196	97.9 ± 5.5
GUDCA	0.01	0.20	0.21	0.208	98.9 ± 4.2
GCDCA	0.10	0.20	0.30	0.300	99.9 ± 4.0
GDCA	0.05	0.20	0.25	0.234	93.7 ± 5.2
GLCA	<0.005	0.20	0.20	0.190	95.1 ± 5.8
TCA	<0.005	0.20	0.20	0.185	92.7 ± 4.6
TUDCA	<0.005	0.20	0.20	0.198	98.9 ± 4.7
TCDCA	<0.005	0.20	0.20	0.192	96.1 ± 5.8
TDCA	<0.005	0.20	0.20	0.180	90.1 ± 4.6
TLCA	<0.005	0.20	0.20	0.181	90.3 ± 4.5

*See Table I for abbreviations.
**$n = 10$.

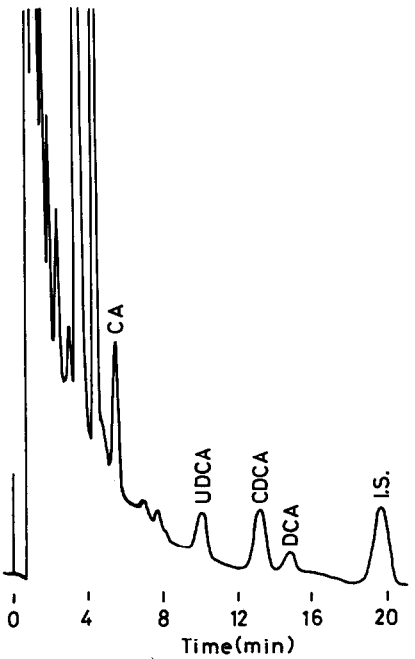

Fig. 7. A chromatogram of free bile acids in serum of a healthy subject. For abbreviations, see Table I.

chromatogram of free bile acids in human serum is illustrated in Fig. 7. The cholic acid peak on the chromatogram represents approximately 500 fmol as an injected amount. The chromatogram with a stable baseline and without leading and tailing is favorable for the determination of bile acids with a quantification limit of 5 pmol per 0.1 ml of serum. Simultaneous determination of bile acids was carried out with serum specimens taken from eight male healthy volunteers. The results obtained are listed in Table III.

It is hoped that the availability of a new method for the simultaneous determination of serum bile acids with satisfactory reliability and sensitivity may provide much more precise knowledge on the metabolic profile of bile acids and may serve as a diagnosis for hepatobiliary diseases.

TABLE III

AMOUNTS OF BILE ACIDS IN SERUM OF HEALTHY SUBJECTS (A—H)

Results are given in nmol/ml.

Bile acid*	A	B	C	D	E	F	G	H
CA	n.d.	n.d.	0.89	0.39	0.36	n.d.**	n.d.	n.d.
UDCA	0.81	0.34	n.d.	0.36	0.30	0.30	0.31	0.16
CDCA	2.09	0.25	1.20	0.28	0.58	0.22	1.50	0.38
DCA	n.d.	0.96	1.12	0.48	0.42	0.34	0.26	0.44
LCA	0.13	n.d.	n.d.	n.d.	0.05	n.d.	n.d.	0.08
GCA	0.84	0.56	0.57	0.86	0.74	0.89	0.24	0.12
GUDCA	0.12	n.d.	0.21	n.d.	0.05	0.05	0.05	n.d.
GCDCA	2.19	1.01	1.90	1.21	3.38	2.87	1.03	1.27
GDCA	n.d.	0.43	1.32	0.15	0.78	0.74	n.d.	0.48
GLCA	0.19	n.d.	0.05	0.05	n.d.	n.d.	n.d.	n.d.
TCA	0.09	n.d.	n.d.	0.06	0.10	0.07	n.d.	n.d.
TUDCA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TCDCA	0.20	n.d.	0.06	0.13	0.27	0.26	0.18	0.17
TDCA	0.13	0.11	0.09	n.d.	n.d.	n.d.	0.12	n.d.
TLCA	n.d.	n.d.	0.05	n.d.	n.d.	0.05	n.d.	n.d.
Total	6.79	3.66	7.41	3.92	6.98	5.74	3.64	3.10

*See Table I for abbreviations.
**n.d. = not detectable (<0.05 nmol/ml).

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Shimadzu Science Foundation and the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., (1979) 461.
2 S. Kamada, M. Maeda and A. Tsuji, J. Chromatogr., 272 (1983) 29.
3 S. Okuyama, N. Kokubun, S. Higashidate, D. Uemura and Y. Hirata, Chem. Lett., (1979) 1443.
4 K. Arisue, Z. Ogawa, K. Kohda, C. Hayashi and Y. Ishida, Japn. J. Clin. Chem., 9 (1980) 104.
5 S. Kamada, M. Maeda, A. Tsuji, Y. Umezawa and T. Kurahashi, J. Chromatogr., 239 (1982) 773.
6 J. Goto, S. Komatsu, N. Goto and T. Nambara, Chem. Pharm. Bull., 29 (1981) 899.
7 J. Goto, N. Goto, F. Shamsa, M. Saito, S. Komatsu, K. Suzaki and T. Nambara, Anal. Chim. Acta, 147 (1983) 397.
8 J. Goto, H. Kato, F. Hasegawa and T. Nambara, Chem. Pharm. Bull., 27 (1979) 1402.
9 J. Goto, K. Suzaki and T. Nambara, Chem. Pharm. Bull., 28 (1980) 1258.
10 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.
11 N.A. Parris, J. Chromatogr., 133 (1977) 273.
12 R. Shaw, J.A. Smith and W.H. Elliott, Anal. Biochem., 86 (1978) 450.
13 C.A. Bloch and J.B. Watkins, J. Lipid Res., 19 (1978) 510.
14 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, J. Chromatogr., 152 (1978) 431.
15 J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Liquid Chromatogr., 3 (1980) 991.
16 J. Goto, H. Kato, K. Kaneko and T. Nambara, J. Liquid Chromatogr., 4 (1981) 1351.