Nuclear Magnetic Resonance Spectrometry and Liquid Chromatography of Two Bile Acid Epimers: Ursodeoxycholic and Chenodeoxycholic Acid

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Abstract □ A specific reverse-phase high-performance liquid chromatographic (HPLC) technique is described for the analysis of bile acids and their conjugates in human serum. Precise quantitation is obtained using UV detection. ^{13}C-NMR spectrometry suggests a structural explanation for the different HPLC retention times of chenodeoxycholic acid, its epimer (ursodeoxycholic acid), and their methyl esters.

Keyphrases □ Ursodeoxycholic acid—HPLC in human serum, ^{13}C-NMR elucidation of structure, methyl ester □ Chenodeoxycholic acid—HPLC in human serum, ^{13}C-NMR elucidation of structure, methyl ester

Determination of individual bile acids in biological fluids by TLC, fluorometry, and GC has been reported (1–3). The glycine and taurine conjugates were determined by TLC; however, the resolution was unsatisfactory. Fluorometry (4) using 3α-hydroxy steroid dehydrogenase allows one to quantitate total bile acids only, while the GC method is tedious. High-performance liquid chromatography (HPLC) offers the possibility of separation and quantitation of individual bile acids without sample derivatization. This method is rapid and sensitive and can be used for many routine applications.

EXPERIMENTAL

Apparatus—A high-performance liquid chromatograph[1] equipped with a 20-μL loop injector[2] and a variable-wavelength absorbance detector[3] set at 201 nm was used. The chromatograms were plotted on a strip-chart recorder[4]. The stainless-steel column (3.9 × 15 mm) was packed with C_{18}-bonded microparticles[5].

Reagents and Solvents—The free bile acids and the taurine and glycine conjugates were obtained commercially[6]. Methyl esters of chenodeoxycholic acid and ursodeoxycholic acid were prepared by treatment of the acids with diazomethane. Further purification was carried out by TLC on silica gel (benzene–acetone–methanol, 85:5:10). UV-grade methanol[7] and double-distilled water were filtered[8] (pore size, 0.5 μm), stored in glass apparatus, and deaerated before use. Perchloric acid[9], analytical reagent grade, was used to adjust the pH of the mobile phase.

Samples—Serum (1 mL) was passed through a C_{18} microparticulate microcolumn[10] fixed onto a syringe. The column was washed with double-distilled water (2 × 4 mL), then by four methanol–water mixtures with increasing concentration of methanol (0–50%) (2 × 2 mL). Bile acids were eluted with pure methanol (3 mL). The filtrate was evaporated under nitrogen at 80°C. The residue was dissolved in 50 μL of double-distilled water, and a 20-μL aliquot was injected onto the column using the injector loop.

No internal standard was used. A 20-μL portion of external standard was also analyzed under the same chromatographic conditions. The standard solution was prepared daily by dissolving weighed amounts of each bile acid in water. The extraction ratio depended on each bile acid, ranging from 95 to 100%.

Chromatographic Conditions—Methanol (76%) in aqueous potassium dihydrogen phosphate (30 mmol/L) was used as the mobile phase. The pH of the mobile phase was adjusted after addition of methanol using perchloric acid (0.02%). The flow rate was 1.2 mL/min. The absorbance was measured at 201 nm (0.02 AUFS). The limit of sensitivity was 1 ng/μL of injected solution.

pK_a Determination—The apparent ionization constants (pK'_a) for ursodeoxycholic and chenodeoxycholic acid and their methyl esters were determinted by potentiometry[11] with a hydrogen electrode at 25°C in methanol–water (70:30, w/w). The pK_a values were calculated by the usual method (5).

^{13}C-NMR Spectra—A Fourier transform ^{13}C-NMR spectrometer[12] was used at probe temperature. Samples were dissolved in methanol-d_4 giving final concentration of 1 mol/L. Shifts from the methanol reference were measured on a 2000-Hz sweep width with 8192 data points (0.488 Hz/resolution point). The spectral parameters were: flip angle 45° (10-μs pulse width); repetition rate, 2.05 s; weighting function for sensitivity enhancement, 0.4 s; decoupling power, 4500 Hz; decoupling frequency, offset 52 (CFT 20 program); noise bandwith, 1000 Hz; and total experiment time, 160 nm (4700 scans). For S.F.O.R.D., the experimental decoupler frequency offset was 42 (cft 20 program) and the noise bandwith was off.

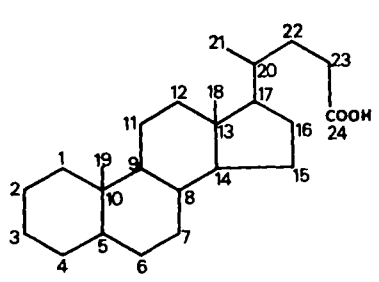
These typical experimental conditions were specially adapted to noise decoupling spectra of steroid molecules. They were determinated empirically for this spectrometer to obtain different signal heights for CH_3, CH_2, CH, and quaternary C. The association of information provided by this method with single frequency off-resonance spectra has improved the assignment of carbon signals in steroids.

RESULTS AND DISCUSSION

Bile acids have a 5β-cholanic acid skeleton and possess one, two, or three hydroxyl groups; they are found in serum as free acids (pK_a 5.5–7.0) and the glycine and taurine conjugates (pK_a 3.5–4.5 and ~2, respectively).

Separation and quantitative analysis of bile acids can be used in the diagnosis and study of hepatic and biliary diseases. Using reverse-phase HPLC, the taurine conjugates are eluted first, followed by the glycine conjugates, and then the free bile acids (6). Ursodeoxycholic acid (I) is eluted earlier than the epimeric chenodeoxycholic acid (II). Figure 1 shows a chromatogram under the described conditions; the retention times of ursodeoxycholic and chenodeoxycholic acids, the calculated capacity factors k', were very different: $k' = 3.4$ for ursodeoxycholic acid (I) and $k' = 11.4$ for chenodeoxycholic acid (II).

This surprising observation led to a study of the physical properties and



Cholanic Acid
(I) Ursodeoxycholic Acid: 3α,7β - dihydroxy 5β - cholanic acid
(II) Chenodeoxycholic Acid: 3α,7α - dihydroxy 5β - cholanic acid

[1] Pump Chromatem M380; Touzart et Matignon, France.
[2] Model 210 sample injection valve; Altex Scientific, Palo Alto, Calif.
[3] Model 450 variable-wavelength detector; Waters Associates.
[4] Servotrace PE 1521; Sefram, Paris.
[5] Lichrosorb RP 18, 5 μm; Merck, Darmstadt, W. Germany.
[6] Calbiochem-Behring Corp.
[7] SDS.
[8] Millipore Corp., Mass.
[9] RP Normapur, Prolabo, France.
[10] Waters Associates.
[11] pH-meter E 516; Titriskop Metrohm, Switzerland.
[12] Varian CFT 20 spectrometer.

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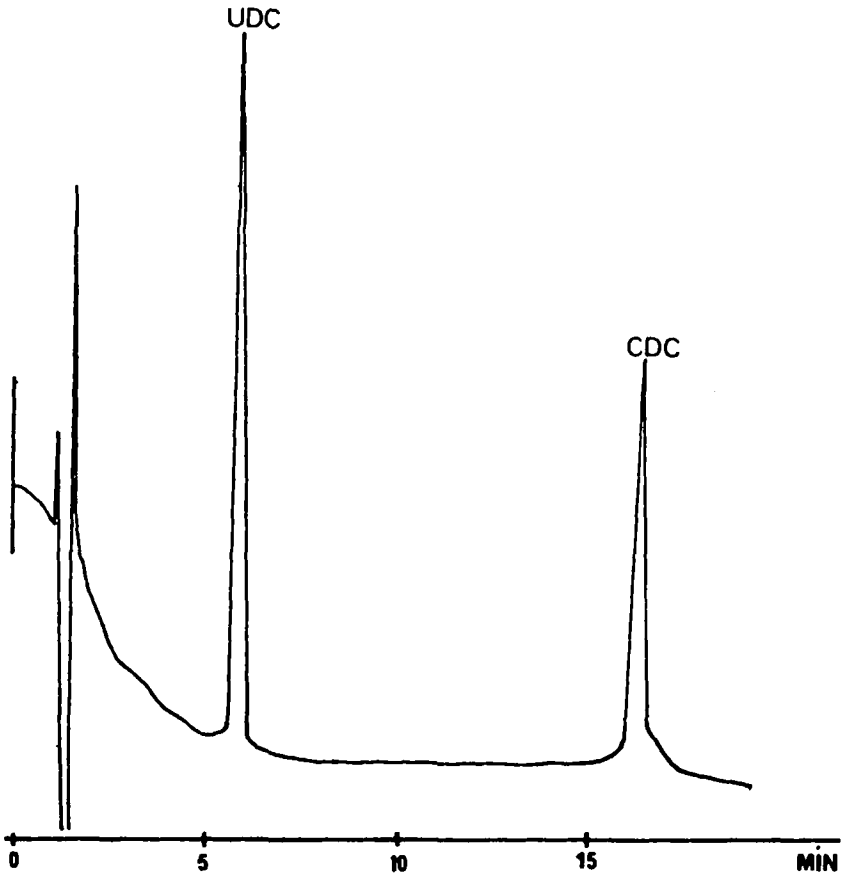

Figure 1—*Chromatogram of ursodeoxycholic acid (UDC) and chenodeoxycholic acid (CDC). Operating conditions were: flow rate, 1.2 mL/min; detector, 201 nm; scale, 0.2 AUFS; sample size, 70 μg/20 μL.*

structural characteristics of these compounds. The melting points and rotations $[\alpha_D]$ of each compound were quite different: ursodeoxycholic acid, mp 203°C, $[\alpha]_D$ +11.5°; chenodeoxycholic acid, mp 143°C, $[\alpha]_D$ +57° (7).

This report describes the pK_a values and the ^{13}C-NMR spectra of these compounds. The pK_a values of ursodeoxycholic acid, chenodeoxycholic acid, and their methyl esters were determined by potentiometry using a hydrogen electrode. The results (Table I) are inconsistent with the physical and chromatographic data in the case of the methyl esters, with the carbonyl function masked; only the hydroxyl group are weakly acidic and the pK_a values are similar. However, the variation of pK_a values in the free acids should be more significant than the observed values obtained by electrochemical methods.

Our hypothesis was that these differences could be explained by a hydrogen bond involving the 7-hydroxyl group of chenodeoxycholic acid. This hydrogen-bonded internal complex could react with the nonpolar stationary phase in liquid chromatography and increase its elution time.

We attempted to show the existence of the hydrogen bond by ^{13}C-NMR spectrometry. The ^{13}C-NMR spectra of common bile acids and their methyl esters have been described and attributions discussed (8, 9) (Table II). Blunt and Stothers (10) describe substitution increments in the steroid carbon skeleton of 5α- and 5β-androstane. With these increments, it is possible to predict the effect of hydroxyl inversion of C-7 on the chemical shifts of neighboring carbon atoms. The corresponding increments were calculated from the published values and are presented in Table III.

The ^{13}C-NMR spectra of ursodeoxycholic acid and its methyl ester have not been described. We assigned carbon signals and compared these with observed chemical shifts using the inversion increment from ursodeoxycholic acid, to obtain the chemical shifts on chenodeoxycholic carbon atoms. Table II compares the values of the observed chemical shifts of ursodeoxycholic acid, chenodeoxycholic acid, and their methyl esters with the calculated chemical shifts of chenodeoxycholic acid and its methyl ester (Fig. 2). Observed and calculated chemical shifts of chenodeoxycholic acid carbons are very similar (deviations, 1 ppm) for all carbons except C-7 (bearing a hydroxyl group) and

Table I—$pK_{a'}$ Values in Methanol–Water (70:30, w/w) at 25°C

Compound	$pK_{a'}$
Ursodeoxycholic acid	6.70
Chenodeoxycholic acid	6.78
Methyl ursodeoxycholate	11.00
Methyl chenodeoxycholate	11.10

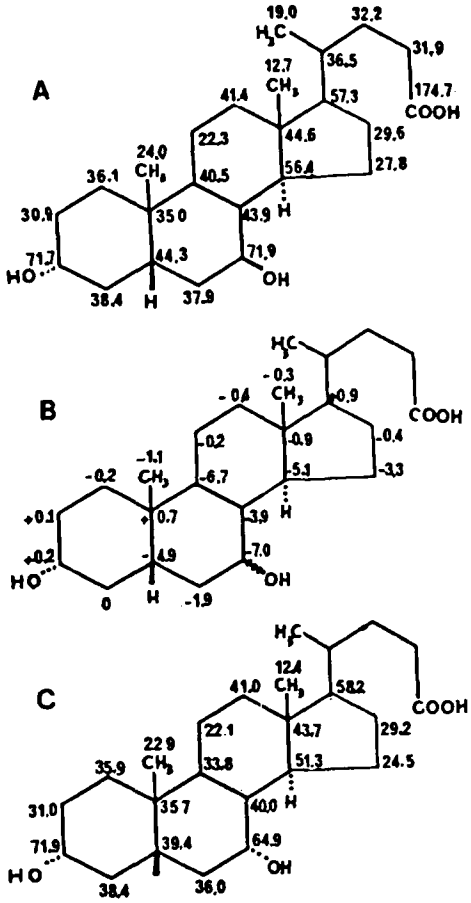

Figure 2—*(A) Observed chemical shifts of ursodeoxycholic acid; (B) increments for 7β → 7α inversion; (C) calculated chemical shifts of chenodeoxycholic acid.*

Table II—Carbon-13 Chemical Shifts of Ursodeoxycholic Acid and Chenodeoxycholic Acid in CH_3OH-d_4 Using Tetramethylsilane as an Internal Standard [a]

	UDC Acid	CDC Acid		Methyl Ester	Methyl Ester CDC	
	7β Obs.	7α Calc.	7α Obs.	UDC 7β Obs.	7α Calc.	7α Obs.
C-1	36.1	35.9	36.5	34.8	34.6	35.1
C-2	30.9	31.0	31.2	30.4	30.5	30.7
C-3	71.7	71.9	72.7	70.9	71.1	71.6
C-4	38.4	38.4	40.3	40.0	40.0	39.2
C-5	44.3	39.4	43.1	43.4	38.5	41.3
C-6	37.9	36.0	36.1	36.9	35.0	34.4
C-7	71.9	64.9	68.9	70.9	63.9	68.2
C-8	43.9	40.0	40.7	42.3	38.4	39.2
C-9	40.5	33.8	33.9	36.9	30.2	32.6
C-10	35.0	35.7	35.8	33.3	34.0	34.9
C-11	22.3	22.1	21.7	21.0	20.8	20.3
C-12	41.4	41.0	41.0	39.1	38.7	39.2
C-13	44.6	43.7	43.6	43.5	42.6	42.4
C-14	56.4	51.3	51.4	54.8	49.7	50.1
C-15	27.8	24.5	24.6	26.7	23.4	23.4
C-16	39.6	29.2	29.1	28.4	28.0	27.9
C-17	57.3	58.2	57.2	55.6	56.5	55.6
C-18	12.7	12.4	12.2	11.9	11.6	11.5
C-19	24.0	22.9	23.4	23.2	22.1	22.6
C-20	36.5		36.6	35.1		35.1
C-21	19.0		18.8	18.2		18.0
C-22	32.2		32.2	30.4		30.7
C-23	31.9		31.9	29.9		30.3
C-24	174.7		175.1	174.7		174.7

[a] UDC = ursodeoxycholic acid; CDC = chenodeoxycholic acid.

C-5 and C-9, which support an important γ-effect from the axial hydroxyl group.

The carbon chemical shifts of the methyl esters also agree closely. Nevertheless, the increments used involve the isolated variation of C-7 configuration and do not take into account the hypothetical intramolecular hydrogen bond between the C-7 hydroxyl and neighboring protons.

Thus, the observed deviations in the chemical shifts of C-4, C-5, C-7, and C-9 as compared with shifts calculated by the increment method may be explained by the existence of a hydrogen bond involving the axial C-7 hydroxyl group of chenodeoxycholic acid and C-4 protons. The values of these deviations agree with a delocalization of σ-bond on a hydrogen bond and an improvement of the γ-axial effect of the C-7 hydroxyl group taking part in a hydrogen bond with C-5 and C-9.

The existence of a hydrogen bond and steric congestion around the C-7 hydroxyl group appears to explain the polarity of these bile acids. Chenodeoxycholic acid appears to be less polar than ursodeoxycholic acid, in agreement with the experimental results obtained by HPLC.

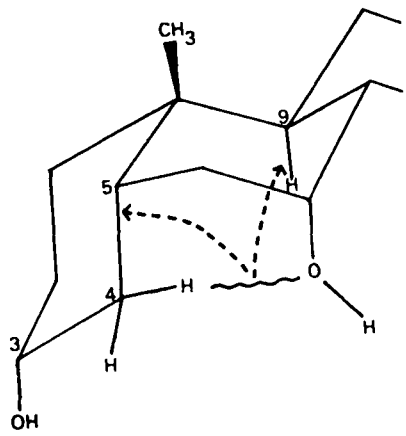

Table III—Substitution Increments in the Steroid Carbon Skeleton: Effect of Hydroxyl Inversion on C-7

Carbon of Steroid Skeleton	Effect of 7αOH [a]	Effect of 7βOH [a]	Effect of 7β → 7α Inversion
C-1	−0.3	−0.1	−0.2
C-2	−0.1	−0.2	+0.1
C-3	−0.1	−0.3	+0.2
C-4	−0.5	−0.5	0
C-5	−7.9	−3.0	−4.9
C-6	+7.6	+9.5	−1.9
C-7	+36.0	+43.0	−7.0
C-8	+4.1	+8.0	−3.9
C-9	−8.5	−1.8	−6.7
C-10	+0.1	−0.6	+0.7
C-11	−0.2	0	−0.2
C-12	−0.5	−0.1	−0.4
C-13	−1.0	+0.1	−0.9
C-14	−5.9	−0.8	−5.1
C-15	−0.5	+2.8	−3.3
C-16	0	+0.4	−0.4
C-17	−0.2	−1.1	+0.9
C-18	−0.3	0	−0.3
C-19	−1.0	+0.1	−1.1

[a] On the chemical shift of neighboring carbon atoms.

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