Radioimmunological determination of serum 3β-hydroxy-5-cholenoic acid in normal subjects and patients with liver disease

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Abstract A radioimmunoassay for the determination of 3β-hydroxy-5-cholenoic acid in human serum has been developed, using 3β-hydroxy-5-cholenoyl-thyroglobulin as immunogen and 3β-hydroxy-5-cholenoylglycyl-^{125}I-histamine as radioactive ligand. The association constant was 6.3×10^8 l/mol. Cross reactivity with other bile acids of human serum was not detectable, but was 5.6% with cholesterol. Serum sample preparation included extraction of 3β-hydroxy-5-cholenoic acid from serum, solvolysis of sulfates, hydrolysis of conjugates, and separation from cholesterol by thin-layer chromatography. Serum concentrations of 3β-hydroxy-5-cholenoic acid were 0.23 ± SD 0.12 μmol/l and 0.21 ± SD 0.09 μmol/l in healthy males and females, respectively. In patients with primary biliary cirrhosis the serum concentration of 3β-hydroxy-5-cholenoic acid and the quotient 3β-hydroxy-5-cholenoic acid over total 3α-hydroxy-bile acids (measured enzymatically) were significantly higher ($P < 0.02$) than in patients with chronic active hepatitis or alcoholic cirrhosis. Analysis of 17 sera with elevated concentration of 3β-hydroxy-5-cholenoic acid by radioimmunoassay and capillary gas–liquid chromatography showed a close correlation ($r = 0.91$, slope = 0.97) between the results of the two methods.—**Minder, E. I., G. Karlaganis, and G. Paumgartner.** Radioimmunological determination of serum 3β-hydroxy-5-cholenoic acid in normal subjects and patients with liver disease. *J. Lipid Res.* 1979. **20:** 986–993.

Supplementary key words radioimmunoassay · bile acids · cholestasis · primary biliary cirrhosis · chronic active hepatitis · alcoholic liver cirrhosis

Ample evidence has established that biosynthesis of the major human bile acids starts with 7α-hydroxylation of cholesterol (1). In patients with cholestasis 3β-hydroxy-5-cholenoic acid, a monohydroxy bile acid with a ring structure identical to that of cholesterol, has been detected (2–5). This finding suggests the existence of a minor pathway of bile acid biosynthesis which begins with oxidation of the cholesterol side chain. Since 3β-hydroxy-5-cholenoic acid constitutes a considerable portion of the bile acids in human meconium (6) and amniotic fluid (7), this pathway may play an important role in fetal life. There is some evidence that synthesis of 3β-hydroxy-5-cholenoic acid continues at a lower rate in the healthy adult (8). The recent detection of 3β-hydroxy-5-cholenoic acid in the urine of healthy adults supports this hypothesis (9). In the serum of healthy subjects, however, this bile acid has not been found (5). This may have been due to insufficient sensitivity of available methods. A sensitive radioimmunoassay for 3β-hydroxy-5-cholenoic acid was, therefore, developed to facilitate further studies of this bile acid in human serum.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. All bile acids were purchased from Supelco Inc., Bellefonte, PA, with the exception of 3β-hydroxy-5-cholenoic acid, which was obtained from Steraloids Inc., Wilton, NH. Lipidex-5000 was purchased from Packard Instruments International S.A., Zurich, Switzerland. TLC aluminium sheets, precoated with silica gel 60 F_{254} (layer thickness 0.2 mm) were obtained from E. Merck A. G., Darmstadt, Fed. Rep. Germany. Thyroglobulin (type II: porcine) was purchased from Sigma Chemical Co., Saint Louis, MO. Freund's complete adjuvant was from Difco Laboratories, Detroit, MI. Spectrapor membrane tubing type 2 was obtained from Spectrum Medical Industries Inc., Los Angeles, CA. Amberlite XAD-2, mesh 100–200 μm and Norit A were purchased from Serva Feinbiochemica, Heidelberg, Fed. Rep. Germany. Dextran, mol wt 70,000, was obtained from Fluka A.G., Buchs, SG, Switzerland. Female

Abbreviations and trivial names: lithocholic, 3α-hydroxy-5β-cholanoic; chenodeoxycholic, 3α,7α-dihydroxycholanoic; deoxycholic, 3α,12α-dihydroxy-5β-cholanoic; and cholic, 3α,7α,12α-trihydroxy-5β-cholanoic acid. BOC, *t*-butoxycarbonyl; DCCI, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; HOBT, 1-hydroxybenzotriazole; GLC, gas–liquid chromatography; RIA, radioimmunoassay; TLC, thin-layer chromatography.

albino guinea pigs were purchased from Madörin, Füllinsdorf, BL, Switzerland. ^{125}Iodine was obtained from Atomic Energy, Canada.

Apparatus

Melting points were determined on a Büchi 510 melting point apparatus (Büchi Flawil, Switzerland) and are uncorrected. Mass spectra were obtained with a Varian MAT CH7A mass spectrometer at an electron energy of 70 eV. ^{13}C-NMR spectra at 25.2 MHz were obtained with the Fourier transform technique using a Varian XL-100 spectrometer.

Preparation of immunogen

3β-Hydroxy-5-cholenoic acid was purified by preparative TLC on silica gel plates developed in acetic acid–carbon tetrachloride–diisopropylether–isopentyl acetate–*n*-propanol–benzene 10:40:60:80:20:20. Thirteen mg (34 μmol) of purified 3β-hydroxy-5-cholenoic acid, 8 mg (68 μmol) of *N*-hydroxysuccinimide, and 9 mg (44 μmol) of *N*,*N*′-dicyclohexylcarbodiimide were dissolved in 0.4 ml of *N*,*N*-dimethylformamide and the reaction was allowed to continue with stirring for 1 hr at room temperature under nitrogen (10). The solution was cooled to 4°C and added under stirring to a solution of 52 mg of thyroglobulin in 2 ml of 0.1 M sodium bicarbonate at 4°C. The mixture was allowed to react for 1 hr and was then dialyzed against 0.1 M ammonium bicarbonate to remove side products. The solution was lyophilized and the antigen was stored at −20°C. Two mg of the conjugate was emulsified in 0.5 ml of 0.9% sodium chloride and 0.5 ml of complete Freund's adjuvant.

Experiments with radioactive chenodeoxycholic acid showed that under the conditions employed 50–60% of the bile acid was incorporated into thyroglobulin. Assuming that the incorporation rate for 3β-hydroxy-5-cholenoic acid was similar, about 217–260 molecules of 3β-hydroxy-5-cholenoic acid were incorporated per molecule of thyroglobulin.

Immunization

Female albino guinea pigs were immunized by intracutaneous injection of 100 μl of antigen (corresponding to about 200 μg of 3β-hydroxy-5-cholenoylthyroglobulin conjugate) at multiple sites on the back. During the first 6 months immunization was repeated monthly, thereafter at 3-month intervals.

Synthesis of 3β-hydroxy-5-cholenoylglycylhistamine

Preparation of glycylhistamine. Histamine dihydrochloride, 1.84 g (10 mmol), and 2.80 ml (20 mmol) of triethylamine were suspended in 10 ml of *N*,*N*-dimethylformamide (DMF), and 1.75 g (10 mmol) of *t*-butoxycarbonyl (BOC)-glycine dissolved in 10 ml of DMF were added. After cooling to 0°C, 2.94 g (20 mmol) of 1-hydroxybenzotriazole (HOBT) were added, followed by 2.27 g (11 mmol) of dicyclohexylcarbodiimide (DCCI) in 10 ml of DMF (11). The mixture was stirred for 2 hr at 0°C and for 18 hr at room temperature. After termination of the reaction with 5 drops of concentrated acetic acid the mixture was centrifuged at 0°C and the supernatant was evaporated to dryness at 30°C.

For removing the BOC protecting group the residue was dissolved in 50 ml of trifluoroacetic acid at room temperature. Five min later the reaction was terminated by the addition of 200 ml of diethylether. After centrifugation, the ether was discarded, and the residue was washed with ether and dried. The crude product was dissolved in 20 ml of methanol–water–chloroform 70:30:10 (by vol) and applied to a Lipidex-5000 column (100 × 2.5 cm id) (12). The column was eluted with the same solvent mixture at a flow rate of 1 ml/min. Fractions of the eluate (5 ml) were collected under monitoring at 206 and 280 nm. The fractions of the first peak were pooled and evaporated to dryness at 40°C.

To obtain the chloride salt, the residue was dissolved in 50 ml of 1% hydrochloric acid and the solution was passed through a column (20 ml) of Dowex 1 (Cl^- form) resin which was eluted with water (100 ml). After evaporation to dryness at 40°C, the combined eluates and washings yielded 1.25 g of glycylhistamine dihydrochloride. The melting point was 262°C. Thin-layer chromatography on silica gel 60 F_{254} (ethyl acetate–methanol–25% ammonia in water 55:35:10); $R_f = 0.35$ (visualized by iodine).

Preparation of 3β-hydroxy-5-cholenoylglycylhistamine. Glycylhistamine dihydrochloride, 120 mg (0.5 mmol), and 0.16 ml (1.1 mmol) of triethylamine were dissolved in 1 ml of DMF. 3β-Hydroxy-5-cholenoic acid, 187 mg (0.5 mmol), dissolved in 2 ml of DMF was added. At 0°C 147 mg (1 mmol) of HOBT in 1 ml of DMF and 113 mg (0.55 mmol) of DCCI in 1 ml of DMF were added and the mixture was stirred for 18 hr at 40°C (11). After adding 3 drops of glacial acetic acid at 0°C the suspension was centrifuged and the supernatant was evaporated to dryness at 40°C. The residue was subjected to chromatography on Lipidex-5000 (12) as described above. The fractions of the second peak were pooled, evaporated to dryness at 40°C, and rechromatographed on Lipidex-5000. The yield was 155 mg of pure 3β-hydroxy-5-cholenoylglycylhistamine. The R_f determined by thin-layer chromatography (conditions as described above) was 0.62. The melting point was 197°C.

Mass spectrum results were: *m/e* 524 (M^+), 506, 491, 467, 413. ^{13}C-NMR spectrum (in dimethylsulfoxide-d_6 at 60°C) showed δ (ppm from TMS) 173.4, 169.2, 141.5, 134.7, 134.3, 120.5, 120.4, 118.8, 116.9, 70.4, 56.6, 55.9, 50.1, 42.6, 42.5, 42.3, 39.7, 36.5–12.0 (14 lines).

Iodination of 3β-hydroxy-5-cholenoylglycylhistamine

Thirty μg of 3β-hydroxy-5-cholenoylglycylhistamine in 30 μl of ethanol was added to 3 μl of phosphate buffer (0.5 M, pH 7.4). To this mixture approximately 2 mCi of ^{125}I was added in a volume of 3 μl. Aliquots (2 μl) of chloramine-T (1 mg/ml water) were added three times at 30-sec intervals (13). The reaction was terminated by the addition of 5 μl of sodium metabisulfite (1 mg/ml water), followed by dilution of the reaction mixture with 1 ml of phosphate buffer (0.01 M, pH 7.4). Subsequently, the reaction mixture was transferred to a small column of Amberlite XAD-2 resin (volume approximately 3 ml). After washing the column with 3 ml of phosphate buffer and 5 ml of water, the 3β-hydroxy-5-cholenoylglycyliodohistamine was eluted with 3 ml of methanol. The methanolic solution was condensed to 0.3 ml and the tracer was further purified by thin-layer chromatography on silica gel using ethyl acetate–benzene–methanol–triethylamine 50:25:25:10 as solvent. The specific activity of 3β-hydroxy-5-cholenoylglycyl-^{125}I-histamine was 6.8 Ci/mmol.

Serum sample preparation

Extraction from serum, solvolysis, and alkaline hydrolysis of bile acids were performed according to the method of Ali and Javitt (14): 3.5 ml of 2,2-dimethoxypropane and 0.4 ml of 1 M hydrochloric acid in methanol were added to 0.5 ml of serum or diluted serum (1:5 in water) and the mixture was kept at −20°C for 0.5 hr and then at room temperature for 3 hr. After centrifugation, the supernatant was collected and the pellet was washed once with 0.5 ml of acetone. The combined supernatant and washing were neutralized with 1 M sodium hydroxide in methanol and brought to dryness at 40°C under nitrogen. The residue was dissolved in 2 ml of 1.25 M sodium hydroxide containing EDTA (13 mM) and boiled for 3 hr at 1 atm. After cooling to room temperature, the mixture was adjusted to pH 1 with 6 M hydrochloric acid. The free bile acids were extracted with 7 ml of chloroform and brought to dryness at 40°C under nitrogen. The residue was dissolved in about 100 μl of chloroform and the bile acids were separated from cholesterol by TLC on silica gel using isopropanol–ethyl acetate–ammonia–water 20:25:6:4 as solvent (15). 3β-Hydroxy-5-cholenoic acid was located by a reference chromatogram; it was eluted from the silica gel with boiling methanol and the solution was brought to dryness. The residue was dissolved in phosphate buffer (16) and appropriate aliquots (20–100 μl) were assayed by RIA.

Radioimmunoassay

The assay procedure was essentially the same as that recently described for cholic acid (16). Briefly, 1 ml of diluted antiserum was mixed with 0.1 ml of serum extract and 0.05 ml of tracer solution (8,000–10,000 cpm). The samples were incubated for 1 hr at 37°C and 0.5 hr at 4°C. Free antigen was adsorbed on dextran-coated charcoal, which was separated by centrifugation. The supernatant was decanted and its radioactivity was counted in a Packard Auto-Gamma spectrometer system, model 5130. Standard curves and serum samples were prepared in duplicate. A linear logit–log transformation was used for analysis. In each assay a blank containing water was included.

Capillary gas–liquid chromatography

In 17 sera, radioimmunologic determination of 3β-hydroxy-5-cholenoic acid was validated by a capillary gas–liquid chromatographic method described previously (17, 18). Total 3α-hydroxy bile acids were determined in all sera by an enzymatic method using purified 3α-hydroxy steroid dehydrogenase (19).

Subjects

The study comprised 20 fasting healthy subjects (10 females, 10 males) aged 23 to 46 years and 50 patients (22 females, 28 males) (Tables 1–3) with various liver diseases. The diagnosis in the patients was established by clinical and laboratory criteria and in 40 of the 50 patients by liver biopsy. Nine patients (all females) had primary biliary cirrhosis (**Table 1**), 20 patients (10 females, 10 males) had chronic active hepatitis (**Table 2**), and 21 patients (3 females, 18 males) had alcoholic liver cirrhosis (**Table 3**).

Statistics

Regression analysis was performed by the method of least squares. The statistical significance of differences between groups was tested by the Mann-Whitney rank test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Titers of antisera

In all six animals immunized with the 3β-hydroxy-5-cholenoyl-thyroglobulin complex, specific antibodies

TABLE 1. Serum total bile acids and serum 3β-hydroxy-5-cholenoic acid in patients with primary biliary cirrhosis

Patient Initials	Sex	Age	Bilirubin	Aspartate Amino-transferase	Alkaline Phosphatase	γ-Globulins	BSP 45-min Retention	Total 3α-Hydroxy-bile Acids	3β-Hydroxy-5-cholenoic Acid
			mg%	*IU/l*	*IU/l*	*%*	*%*	*μmol/l*	*μmol/l*
M.H.	f	39	1.7	76	299	38	31	18	1.4
R.G.	f	58	3.7	57	105	29	40	20	2.2
H.E.	f	54	2.8	52	336	18	40	34	1.5
S.C.	f	69	2.2	43	189	17	52	51	1.7
J.L.	f	68	2.7	46	550	29	33	51	0.9
Z.Z.	f	39	2.7	66	217	16		78	3.0
H.K.	f	65	1.5	36	181	34		128	1.6
B.G.	f	58	7.5	32	173	26		136	1.4
M.H.	f	33	9.5	122	115	14		246	6.8
Mean ± SD								84 ± 74	2.3 ± 1.8
Range								18–246	0.9–6.8

could be raised. Titers ranged from 1:500 to 1:600. The antiserum with the highest titer (1:600) was used for further studies.

Sensitivity

The standard curves (means ± SD of 12 consecutive determinations) plotted in the logit–log transformation were linear for values of B/B_0 ranging from 0.1 to 0.9 (**Fig. 1**). The lower limit of sensitivity (defined as $B/B_0 = 0.9$) was 0.6 pmol, the upper limit ($B/B_0 = 0.1$) was 37 pmol. The Scatchard plot revealed a linear relationship between the quotient bound to free antigen versus bound antigen (**Fig. 2**). The association constant was calculated to be $6.3 \pm 0.6 \times 10^8$ l/mol and the binding capacity to be 0.52 μmol/l of undiluted serum.

Specificity of antisera

Cross reactivities with different bile acids and cholesterol were tested and calculated as recently described (16). With the major human bile acids they were below 0.1%. With cholesterol a cross reactivity of 5.6% was found (**Table 4**). It was, therefore, necessary to eliminate cholesterol during serum sample preparation. This was accompanied by thin-layer chromatography (see Methods).

TABLE 2. Serum total bile acids and serum 3β-hydroxy-5-cholenoic acid in patients with chronic active hepatitis

Patient Initials	Sex	Age	Bilirubin	Aspartate Amino-transferase	Alkaline Phosphatase	γ-Globulins	BSP 45-min Retention	Total 3α-Hydroxy-bile Acids	3β-Hydroxy-5-cholenoic Acid
			mg%	*IU/l*	*IU/l*	*%*	*%*	*μmol/l*	*μmol/l*
F.R.	m	21	0.9	360	43	16	29	13	0.64
W.E.	m	58	1.4	34	44	33	22	16	0.49
Z.E.	m	57	2.3	42	114	26	30	16	0.21
G.K.	f	59	1.1	20	68	30	18	19	0.53
L.H.	f	40	1.8	176	84	13	32	19	0.52
F.F.	m	80	1.0	39	56	43	24	21	0.23
S.S.	f	54	1.0	173	62	19	47	25	0.56
M.S.	f	33	0.9	31	26	25	14	32	0.02
R.L.	f	60	1.1	42	37	36	54	32	0.09
E.J.	f	43	1.8	74	106	37	37	33	0.43
S.J.	f	58	2.5	21	44	29	—	39	0.38
F.A.	m	63	1.5	129	134	21	26	41	0.02
R.F.	f	54	0.3	35	68	24	13	42	0.02
C.C.	m	59	2.9	120	58	23	50	43	0.70
S.H.	f	46	1.2	34	45	25	32	44	0.19
S.G.	m	49	1.1	50	39	22	28	44	0.01
D.R.	f	67	14.8	418	460	29	37	68	0.71
F.V.	m	42	1.3	47	175	19	17	76	0.01
N.P.	m	62	1.4	131	38	24	35	113	0.21
R.G.	m	54	3.9	250	85	24	46	213	0.69
Mean ± SD								48 ± 46	0.33 ± 0.26
Range								13–213	0.01–0.71

TABLE 3. Serum total bile acids and serum 3β-hydroxy-5-cholenoic acid in patients with alcoholic liver cirrhosis

Patient Initials	Sex	Age	Bilirubin	Aspartate Amino-transferase	Alkaline Phosphatase	γ-Globulins	BSP 45-min Retention	Total 3α-Hydroxy-bile Acids	3β-Hydroxy-5-cholenoic Acid
			mg%	*IU/l*	*IU/l*	%	%	*μmol/l*	*μmol/l*
G.S.	m	61	0.8	21	98		20	10	0.24
R.G.	m	56	1.5	21	66	15	30	16	0.11
H.HJ.	m	53	0.8	12	51	22	28	21	0.08
K.M.	m	59	2.5	17	38	18	53	26	0.22
M.A.	m	47	1.2	79	117	22	—	27	0.55
E.H.	m	43	1.1	22	104	—	—	27	0.63
S.E.	m	45	0.7	42	57	21	15	32	0.96
H.W.	m	60	0.7	103	104	24	21	33	0.08
C.M.	m	50	1.5	59	122	31	43	34	1.20
K.J.	m	60	14.1	67	69	40	56	36	0.95
M.J.	m	62	1.0	27	45	27	30	37	0.14
K.H.	m	46	4.9	138	45	—	33	38	0.22
D.J.	f	53	2.8	35	37	24	41	46	0.03
M.A.	f	39	1.7	87	128	22	—	66	0.72
vA.E.	f	59	1.1	20	96	27	24	70	0.10
D.A.	m	59	3.5	41	150	24	25	75	0.78
G.P.	m	41	1.9	36	46	24	41	84	0.86
K.H.	m	49	3.9	66	145	54	—	123	0.93
G.W.	m	59	1.9	26	80	41	34	140	0.09
B.R.	m	41	6.4	18	97	26	46	147	0.10
H.F.	m	54	2.4	33	64	30	40	153	0.33
Mean ± SD								59 ± 45	0.44 ± 0.38
Range								10–153	0.03–1.20

Precision of assay

Intra- and interassay variation was determined with both normal serum and serum containing an elevated level of 3β-hydroxy-5-cholenoic acid. Coefficients of variation ranged between 12 and 20% (**Table 5**).

Recovery

Recovery, which was estimated by adding various amounts of 3β-hydroxy-5-cholenoic acid to normal human serum, ranged from 100 to 111% (**Table 6**).

Influence of sample volume

The extract of a serum sample with an elevated 3β-hydroxy-5-cholenoic acid concentration was diluted in phosphate buffer so that 0.1-ml aliquots corresponded to 7.8, 15.6, 31.3, and 62.5 nl of serum. The amounts of 3β-hydroxy-5-cholenoic acid measured were linearly related to the volume assayed (**Fig. 3**). This indicated the absence of unspecific interferences which were not detectable by measurements of cross reactivities.

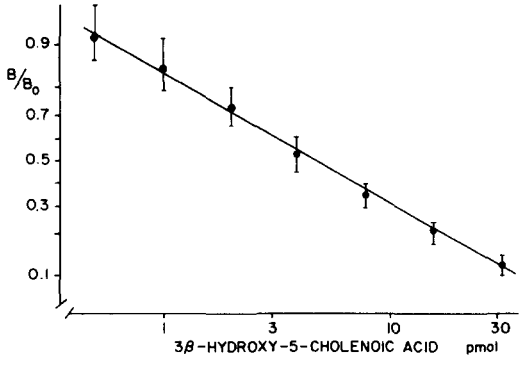

Fig. 1. Standard curve of 3β-hydroxy-5-cholenoic acid. Logit B/B_0 (ordinate) is plotted against the logarithm of the standards (abscissa). Each point represents the mean ± SD from 12 determinations performed at different days over a period of 3 months.

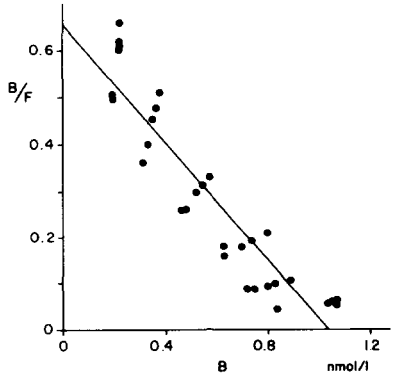

Fig. 2. Scatchard plot of 3β-hydroxy-5-cholenoic acid binding with antiserum. Abscissa: bound antigen (B). Ordinate: Ratio of bound to free antigen (B/F). Regression line: $y = -0.63x + 0.66$.

TABLE 4. Cross reactivities of antiserum with various bile acids and with cholesterol

Compound	Percent Cross Reactivity
3β-Hydroxy-5-cholenoic acid	100
Taurocholic acid	<0.05
Glycocholic acid	<0.05
Taurochenodeoxycholic acid	<0.05
Glycochenodeoxycholic acid	<0.05
Glycodeoxycholic acid	<0.01
Glycolithocholic acid	<0.01
Cholesterol	5.6

Validation

Serum concentrations of 3β-hydroxy-5-cholenoic acid measured by radioimmunoassay in 17 specimens (range: 1.5–16.3 μmol/l) were linearly correlated with the respective values obtained by capillary gas–liquid chromatography ($r = 0.91$). The intercept of the regression line was not significantly different from zero, and the slope (0.97) was not significantly different from one (**Fig. 4**).

Serum concentrations of 3β-hydroxy-5-cholenoic acid

Healthy subjects had fasting serum concentrations of 3β-hydroxy-5-cholenoic acid ranging from 0.08 to 0.45 μmol/l. There was no significant difference between females and males (**Table 7**).

Patients. The serum concentrations of 3β-hydroxy-5-cholenoic acid measured in 50 patients with various liver diseases are given in Tables 1–3. They were increased above the mean plus two standard deviations of healthy controls in all 9 patients with primary biliary cirrhosis, in 8 of 20 patients with chronic active hepatitis, and in 9 of 21 patients with alcoholic liver cirrhosis. The mean concentration of 3β-hydroxy-5-cholenoic acid was significantly higher ($P < 0.001$) in the group of patients with primary biliary cirrhosis (2.3 μmol/l; range 0.9–6.8) than in the groups with chronic active hepatitis (0.33 μmol/l; range 0.01–0.71), and alcoholic liver cirrhosis (0.44 μmol/l; range 0.03–1.20). No significant difference was found between chronic active hepatitis and alcoholic cirrhosis. Serum total 3α-hydroxy bile acids were elevated in all patients studied, but in contrast to 3β-hydroxy-5-cholenoic acid no significant difference was found between any of the three groups of patients (Tables 1–3). There was a small but significant correlation between the concentrations of total 3α-hydroxy bile acids and 3β-hydroxy-5-cholenoic acid in serum when the results of all three groups were analyzed together ($r = 0.47$, $n = 50$, $P < 0.05$). When the groups were analyzed separately a significant correlation was found in the group of patients with primary biliary cirrhosis ($r = 0.77$, $n = 9$, $P < 0.01$), but there was no significant correlation in the patients with chronic active hepatitis ($r = 0.16$, $n = 20$, $P > 0.05$) or alcoholic cirrhosis ($r = -0.02$, $n = 21$, $P > 0.05$). The ratio between 3β-hydroxy-5-cholenoic acid and total 3α-hydroxy bile acids in serum ranged from 0.01 to 0.11 (mean 0.04) in primary biliary cirrhosis, from 0.0001 to 0.05 (mean 0.01) in chronic active hepatitis, and from 0.0006 to 0.04 (mean 0.01) in alcoholic liver cirrhosis. The difference between primary biliary cirrhosis and the two other groups was statistically significant ($P < 0.02$).

TABLE 5. Reproducibility of the assay

Number of Determinations	3β-Hydroxy-5-cholenoic Acid Concentration in Serum	Coefficient of Variation
	μmol/l	%
Intraassay variation		
5	12.1 ± 1.8[a]	15.2
5	0.4 ± 0.07	17.1
Interassay variation		
5	3.2 ± 0.4	12.4
5	0.3 ± 0.06	20.0

[a] Mean ± SD.

TABLE 6. Recovery experiments

3β-Hydroxy-5-cholenoic Acid Added to 1 ml of Serum	3β-Hydroxy-5-cholenoic Acid Recovered	Percent
nmol	*nmol*	
0	0.700[a]	
1.0	1.808	111
2.0	2.746	102
3.0	3.706	100

[a] Values represent means of duplicate determinations.

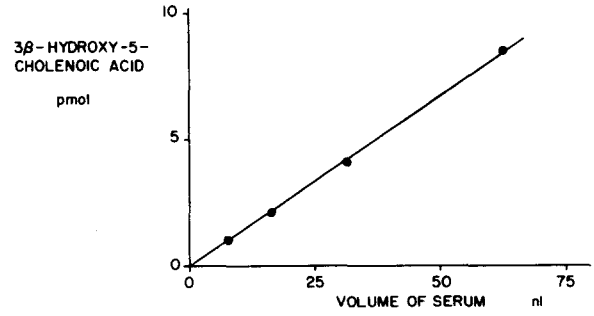

Fig. 3. Determination of 3β-hydroxy-5-cholenoic acid in different dilutions of a serum sample. Abscissa: volume of serum corresponding to 100 μl of dilution. Ordinate: amount of 3β-hydroxy-5-cholenoic acid measured.

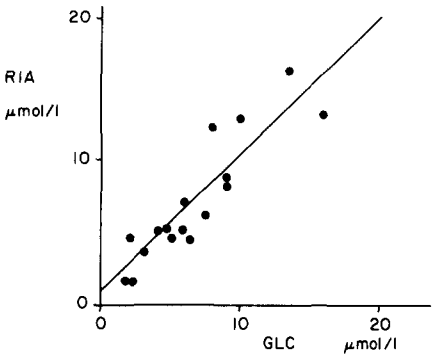

Fig. 4. Comparison of radioimmunologic (RIA) with GLC determination of 3β-hydroxy-5-cholenoic acid in 17 serum specimens from patients with liver disease. Regression line: $y = 0.97x + 0.56$ ($r = 0.91$; $n = 17$).

DISCUSSION

This paper describes a radioimmunoassay for 3β-hydroxy-5-cholenoic acid which permits measurement of this monohydroxy bile acid with greater sensitivity than methods hitherto available. The approach used in the development of this assay is similar to that of Spenney and co-workers (20) who first used a ^{125}I-labeled bile acid derivative as tracer for measurement of cholic acid. The amount of added 3β-hydroxy-5-cholenoic acid required to cause a 10% decrease in the binding of tracer to antiserum was 0.6 pmol. When 0.5 ml of serum was analyzed, the limit of sensitivity was 0.02 μmol/l. This sensitivity was sufficient for measurement of 3β-hydroxy-5-cholenoic acid in serum of fasting healthy subjects. Although cross reactions with the major human bile acids were not detectable, cross reaction with cholesterol which has the same ring structure as 3β-hydroxy-5-cholenoic acid was 5.6%. This necessitated the elimination of cholesterol from serum by thin-layer chromatography. Possible intermediates that could react with the antibody are hydroxycholesterols that are hydroxylated at the side chain. However, these compounds are less polar than 3β-hydroxy-5-cholenoic acid and it can, therefore, be assumed that they were separated from 3β-hydroxy-5-cholenoic acid by thin-layer chromatography prior to RIA.

TABLE 7. 3β-Hydroxy-5-cholenoic acid in serum of fasting healthy subjects

Subjects	Number of Determinations	Age	3β-Hydroxy-5-cholenoic Acid in Serum
		yr	*μmol/l*
Females	10	23–46	0.21 ± 0.09[a] (0.09–0.33)[b]
Males	10	23–44	0.23 ± 0.12 (0.08–0.45)

[a] Mean ± SD.
[b] Range.

Since a large fraction of 3β-hydroxy-5-cholenoic acid in serum is not only conjugated at the carboxyl group but is also sulfated at the hydroxyl group (6), measurement of total 3β-hydroxy-5-cholenoic acid requires solvolysis of sulfates and hydrolysis of conjugates. Alkaline hydrolysis was preferred to enzymatic hydrolysis in order to split methyl esters which may originate during solvolysis (14) and which may not be cleaved by the enzyme cholylglycine hydrolase (21).

Validation of radioimmunoassays that are primarily developed because of insufficient sensitivity of available chemical methods is difficult. By using sera with elevated 3β-hydroxy-5-cholenoic acid, however, it was possible to validate our radioimmunoassay by capillary GLC. Serum concentrations of 3β-hydroxy-5-cholenoic acid measured by radioimmunoassay and by GLC were in close agreement with each other. The intercept of the regression line correlating the values of the two methods was not significantly different from zero. This finding excludes a significant systematic error due to unknown cross reactions that may lead to overestimation of 3β-hydroxy-5-cholenoic acid by radioimmunoassay. However, in the range of normal values, small overestimations of this type cannot be excluded with certainty.

Hitherto, 3β-hydroxy-5-cholenoic acid has not been detected in sera of healthy subjects. The most likely explanation for the failure to find this bile acid in normal serum is insufficient sensitivity of the methods employed for analysis. In spite of previous negative findings one must postulate the presence of 3β-hydroxy-5-cholenoic acid in serum of healthy subjects because up to 0.8 μmol of this bile acid is excreted daily into the urine (9). These observations together with our finding of 0.2 μmol/l of 3β-hydroxy-5-cholenoic acid in serum of healthy subjects support the existence of a minor pathway of bile acid biosynthesis that leads to the formation of 3β-hydroxy-5-cholenoic acid in healthy man.

In agreement with previous studies using GLC, elevations of serum 3β-hydroxy-5-cholenoic acid were found in patients with various liver diseases. Although such elevations were found in all three types of liver disease investigated in the present study, they were more frequently observed in patients with primary biliary cirrhosis (100%) than in patients with chronic active hepatitis (40%) or alcoholic liver cirrhosis (43%). In addition, the mean value of serum 3β-hydroxy-5-cholenoic acid was significantly higher in primary biliary cirrhosis (2.3 μmol/l) than in the two other groups (0.33 and 0.44 μmol/l). By contrast, no significant differences were found when the respective values for total 3α-hydroxy bile acids in serum were compared. These findings may indicate that 3β-

hydroxy-5-cholenoic acid in serum is mainly elevated in cholestatic liver disease as demonstrated in our study by the group of patients with primary biliary cirrhosis. This contention is supported by a significantly higher ratio of 3β-hydroxy-5-cholenoic acid over total 3α-hydroxy bile acids in primary biliary cirrhosis. Obviously, studies must be performed on a larger number of patients and must be extended to other types of hepatobiliary disease in order to evaluate the clinical significance of an elevated serum level of 3β-hydroxy-5-cholenoic acid. Hitherto, such studies have been hampered because of methodological difficulties. It is hoped that the radioimmunoassay described here will facilitate studies of the role of 3β-hydroxy-5-cholenoic acid in cholestatic liver disease.

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