notes on methodology

Radioimmunoassay of chenodeoxycholic acid-3-sulfate in urine

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Summary A sensitive and specific radioimmunoassay (RIA) for the measurement of urinary total chenodeoxycholic acid-3-sulfate (SCDCA) was developed and the accuracy was confirmed. SCDCA bound to bovine serum albumin as the antigen and emulsified with Freund's complete adjuvant was injected into rabbits. The antiserum obtained was capable of binding 75% of [11,12-^{3}H]SCDCA at 1:1000 dilution. The percentage of bound radioactivity decreased linearly with logarithmic increases in unlabeled SCDCA, from 8 to 200 pmol/ml. The antiserum showed an extremely high specificity for SCDCA (free and conjugated), and the values determined by RIA indicated a close correlation with those found by gas-liquid chromatography. The daily urinary SCDCA level was determined using SCDCA-RIA in 12 disease-free humans and 74 patients with chronic liver diseases. In the normal subjects the daily urinary SCDCA level was 0.74 ± 0.83 mg/day and increased levels were evident in all groups with chronic liver diseases. The daily urinary SCDCA level corresponds closely with the extent of hepatic dysfunction.—**Matsuoka, M., and M. Okumura.** Radioimmunoassay of chenodeoxycholic acid-3-sulfate. *J. Lipid Res.* 1988. **29:** 523-526.

Supplementary key words urinary sulfated bile acid • chronic liver disease

With the development of new methods for analyzing bile acids, many reports on measurements of urinary bile acids have appeared (1-9). However, these procedures mainly involve the use of gas-liquid chromatography (GLC) or gas-liquid chromatography-mass spectrometry (GLC-MS) and are too complicated and time-consuming for clinical application. Furthermore, because 30-80% of the urinary bile acids are sulfated conjugates (1-9), initial solvolysis is required for the determination.

Since Simmonds et al. (10) established a radioimmunoassay (RIA) system for the conjugated cholic acid, studies on urinary bile acid using RIA have been reported (8, 11-17) including conjugated cholic acid (8), sulfated glycolithocholic acid (8), and chenodeoxycholic acid (17).

The major components of urinary bile acids are cholic and chenodeoxycholic acids, the latter existing in significantly more sulfated conjugate forms than those of the former (1-3, 8, 9).

We have developed a new RIA for chenodeoxycholic acid-3-sulfate (SCDCA), one of the major components of urinary sulfated bile acids, and have investigated the clinical significance of urinary SCDCA in patients with chronic liver diseases.

MATERIALS AND METHODS

SCDCA

CDCA was obtained from Sigma Chemical Co. (St. Louis, MO) and exceeded 99% purity determined by TLC using chloroform-methanol-acetic acid 40:10:1. SCDCA was synthesized after the 7α-hydroxide group was protected as 7α-formate, according to Tserng and Klein (18). The crystalline solid obtained was 75% SCDCA and 25% CDCA and NaCl as determined by nuclear magnetic resonance spectroscopy and atomic absorption spectrochemical analysis. SCDCA was purified to greater than 99% by TLC.

Immunization

The SCDCA-BSA complex was prepared by the method of Erlanger et al. (19, 20). The procedure was as follows: 28 mg of SCDCA was dissolved in 0.28 ml of dioxane. To this solution 40 μl of tributyramine and 12 μl of isobutylchloroformate were added with stirring for 20 min at room temperature. The mixture was added to 2.8 ml of water with 100 mg of bovine serum albumin (BSA) (Sigma Chemical Co.), 20 ml of dioxane, and 1.5 ml of dimethylformamide, and was stirred at room temperature for 2 hr. This solution was dialyzed against running water overnight.

The pH was adjusted to 4.5; the sediment was dissolved in water, evaporated in vacuo, and used as the antigen. The number of steroid molecules linked to BSA molecules was calculated by the method of Erlanger et al. (20), which showed that the antigen had 18 mol of SCDCA per mol of BSA. Antiserum was prepared by the method of Simmonds et al. (10). Two mg of SCDCA-BSA in 0.5 ml of saline was emulsified with 1.0 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). This mixture was given subcutaneously into the dorsum and each of the four legs of a rabbit at 2- or 3-week intervals.

Four months later, antiserum with 75% binding of added radioactive tracer at 1:1000 dilution was obtained.

Tracer

[11,12-^{3}H]SCDCA was synthesized by the method of Parmentier and Eyssen (21) from [11,12-^{3}H]CDCA (New En-

Abbreviations: RIA, radioimmunoassay; SCDCA, chenodeoxycholic acid-3-sulfate; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; SLCA, sulfated lithocholic acid.

gland Nuclear Co., Boston, MA) and purity exceeded 99% as determined by TLC.

Radioimmunoassay

Radioimmunoassay was carried out by our own procedures (16). Briefly, the assay system consisted of 50 μl of unlabeled SCDCA (2–1000 pmol/ml of 0.05 M borate buffer) as standard or unknown urinary samples (diluted adequately), 200 μl of antiserum (dilution 1:1000 with buffer I), and 50 μl of [11,12-^{3}H]SCDCA (6000–7000 dpm/50 μl of 0.05 M borate buffer). The mixture was incubated at 42°C for 20 min. To the reaction mixture was added 300 μl of ammonium sulfate solution (60% w/v, saturated solution); the preparation was left at room temperature for 10 min, and then centrifuged at 3000 rpm for 10 min. The supernatant was decanted into a scintillation tube containing 6.0 ml of ACS II scintillation fluid (Amersham Co., Arlington Heights, IL). Radioactivity was measured by a Tri-Carb 300 CD scintillation counter (Packard Instruments Co., Downers Grove, IL). Borate buffer (0.05 M) was prepared by dissolving 31.0 g of boric acid and 2.625 g of NaOH in 4.0 l of boiling water and diluted to volume. The pH was adjusted to 8.0. Buffer I consisted of 60 mg of BSA and 50 mg of rat γ-globulin dissolved in 100 ml of the 0.05 M borate buffer. As in a previous study (16), buffer I was used instead of bile acid-free serum with an incubation time of 20 min. Saturated ammonium sulfate solution for separating free and bound bile acids was used, with a separating time of 10 min. The time required was less than 1 hr and the total quantity of liquid was 700 μl, less than that reported in other studies (8, 11–15, 17).

Bound radioactivity was calculated by subtracting the percent activity in the supernatant in the presence of antibody from the 100% free value for the supernatant in the absence of antibody.

GLC

We measured SCDCA in urine samples using GLC by the method of Makino et al. (2) in order to estimate the correlation of the present RIA methodology and GLC. The procedure was as follows: a urine sample was percolated through 10 g of Amberlite XAD-2 column, and bile acids were eluted with methanol. The elute was evaporated to dryness, and the residue was applied to 4 g of Sephadex LH-20 in a column. The sulfate fraction was solvolyzed and hydrolyzed with 15 ml of 15% NaOH for 4 hr at 120°C. The free bile acids were extracted with ether and methylated with diazomethane. The methyl esters were converted to the trifluoroacetate derivatives which were then quantitatively analyzed by GLC, model GC-2C (Shimadzu Manufacturing Co., Kyoto, Japan) with a hydrogen flame detector. The column was 1.5% QF-1 with Chromosorb W as a support in a glass U-tube 150 cm in length and 4 mm in diameter. The column temperature was 215°C.

Subjects

Twelve disease-free humans, 10 patients with chronic inactive hepatitis, 13 with chronic active hepatitis, 27 with compensated hepatic cirrhosis, and 24 with decompensated hepatic cirrhosis were studied. Twenty four-hr urine samples were stored at −20°C until analysis. All subjects were Japanese.

RESULTS

Specific binding of SCDCA to serum

Approximately 75% of [11,12-^{3}H]SCDCA was bound in a 1:1000 dilution of antiserum. The percentage of bound radioactivity decreased linearly when increasing the dilution of antiserum on a logarithmic scale from 1:500 to 1:3000. The percentage of bound radioactivity decreased linearly, with a logarithmic increase in the unlabeled SCDCA concentration from 8 pmol/ml to 200 pmol/ml (**Fig. 1**).

Specificity

The relative amounts of various bile acids required to displace 50% of the bound [11,12-^{3}H]SCDCA are shown in **Table 1**. This SCDCA antiserum possessed an extremely high specficity for SCDCA, glycoSCDCA and tauroSCDCA. There was negligible binding with lithocholic acid-3-sulfate (SLCA), glycoSLCA, and tauroSLCA.

Accuracy and precision of assay

Reproducibility. Intra-assay variation was tested by performing 10 replicate measurements of each of three samples

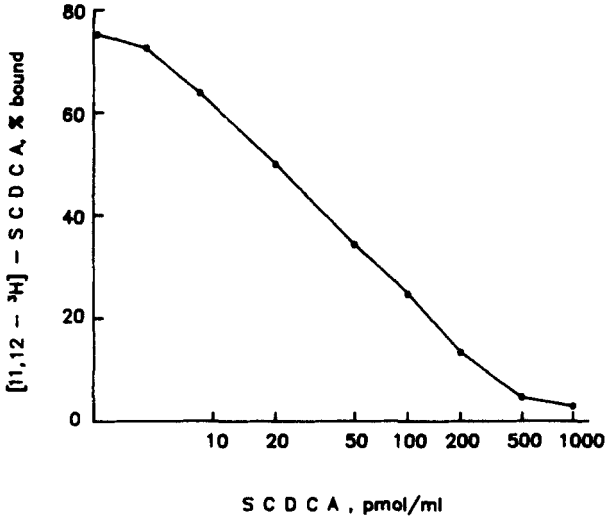

Fig. 1. Standard curve for radioimmunoassay of SCDCA. Radioimmunoassay calibration diagram (antibody dilution 1:1000) showing percentage antibody-bound [11,12-^{3}H]SCDCA in relation to amount (log scale) of SCDCA present in assay.

TABLE 1. Relative binding of various bile acids in radioimmunoassay of SCDCA

Bile Acid	Relative Amount Required to Displace 50% of Bound Tracer
	pmol
Chenodeoxycholic acid-3-sulfate	1
Glycochenodeoxycholic acid-3-sulfate	1
Taurochenodeoxycholic acid-3-sulfate	1
Lithocholic acid-3-sulfate	500
Glycolithocholic acid-3-sulfate	15
Taurolithocholic acid-3-sulfate	40
Chenodeoxycholic acid	> 10000
Glycochenodeoxycholic acid	> 10000
Taurochenodeoxycholic acid	> 10000
Cholic acid	> 10000
Deoxycholic acid	> 10000
Lithocholic acid	> 10000
Ursodeoxycholic acid	> 10000

with concentrations 40, 92, and 146 pmol/ml, respectively. We found that the coefficient of variation (CV) of estimates for each sample was less than 5%. Interassay variation was assessed from four estimates at intervals of 1 week for two samples (59 and 180 pmol/ml, respectively), and the CV of estimates for each sample was less than 10%. These results indicate the excellent reproducibility of this system.

Dilution test. Two urinary samples (84 and 162 pmol/ml) were diluted with 1, 3, and 7 volumes of 0.05 M borate buffer. Good linearities were observed up to an eightfold dilution.

Recovery test. Recovery was studied by the addition of various amounts of SCDCA (40–200 pmol/ml) to two urinary samples, and was tested by this method. The results indicated recoveries, ranging from 90 to 112%.

Assay of human urine

An SCDCA standard curve was used for the assay of human urine. Fifteen urine samples were measured by RIA and GLC. As indicated in **Fig. 2,** the values measured by RIA showed a close correlation to those determined by GLC, and the coefficient of this relationship was estimated to be 0.95 ($P < 0.001$).

In 12 normal subjects, the SCDCA level in the daily urine was 0.74 ± 0.83 (mg/day, mean ± SD) (**Fig. 3**). The upper limit of the normal (mean ± 2SD) was 2.4 mg/day. In patients with various liver diseases, the urinary SCDCA was elevated. The SCDCA levels were 2.33 ± 2.03 in those with a chronic inactive hepatitis, 5.03 ± 5.73 in cases of chronic active hepatitis, 7.62 ± 6.35 in those with a compensated hepatic cirrhosis, and 15.97 ± 10.82 in patients with a decompensated hepatic cirrhosis. The quantities of SCDCA in urine did not correlate with abnormalities in routine biochemical tests of hepatic or renal function.

DISCUSSION

For the measurement of sulfated bile acids, GLC and GLC–MS have been the approaches used. These methods are time-consuming, while RIA is a more convenient method to determine sulfated bile acids, especially when small quantities are available for analysis.

Cowen et al. (15) established an RIA for glycoSLCA in 1977 and Wildgrube et al. (8) reported an RIA for glycoSLCA in urine in 1983. RIA for SCDCA, a major component of urinary sulfated bile acid, has apparently not been reported.

In the present study, the antiserum showed an extremely high specificity for SCDCA (free and conjugated). There was negligible binding with SLCA (free and conjugated), which is 0–10% of the amount of SCDCA in urine. Conjugated SCDCA displaced the radioactive tracer in the same manner as did the free SCDCA. Thus, it was considered that the antigenic structure of SCDCA-BSA compound was similar to that of conjugated SCDCA as well as that of free SCDCA.

The present assay system using a specific antiserum makes feasible detection of 8 pmol of SCDCA in 1.0 ml of urine. Good reproducibility and recovery were also obtained. In addition, the reliability of this assay system was supported by the finding of a high correlation between the values obtained by RIA and GLC. The RIA values were slightly higher.

Observed SCDCA levels in daily urine by this assay as well as by GLC–MS (3, 9) and RIA (17) were 4–5 times higher than those found by GLC (1, 2, 6, 7), probably reflecting the differences in methodology.

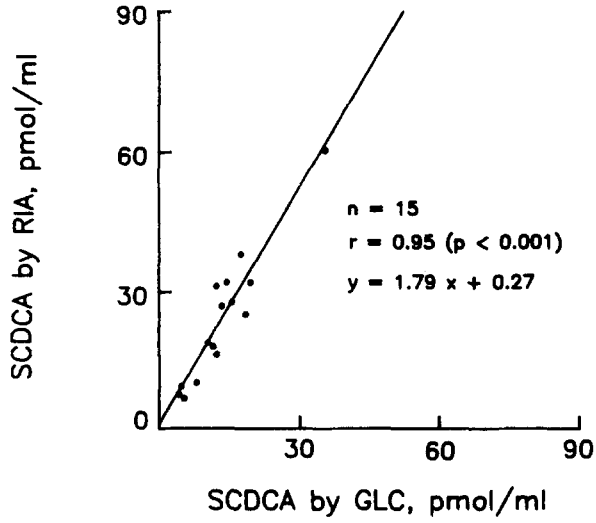

Fig. 2. Correlation between urinary SCDCA values by radioimmunoassay (RIA) and gas-liquid chromatography (GLC).

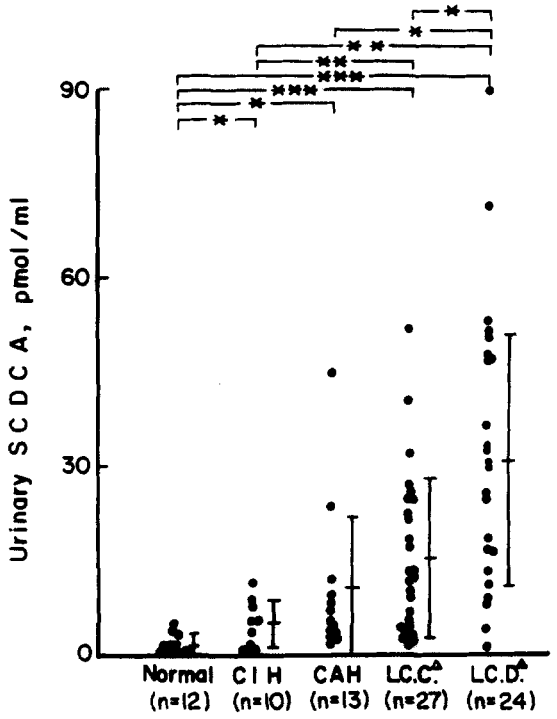

Fig. 3. SCDCA level in daily urine from normal subjects and patients with various liver diseases; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; (△), cirrhosis of the liver, compensated; (▲), cirrhosis of the liver, decompensated.

There were significantly higher levels of SCDCA in urine of patients with chronic liver diseases, as compared to levels in urine from normal subjects. SCDCA levels in daily urine correspond closely with the severity of hepatic dysfunction, but there was some overlap in subjects with chronic liver disease.

Nakamura et al. (7) considered that urinary sulfated bile acids could be a more sensitive index than conventional liver function tests for the prognosis of liver cirrhosis. These results suggest that determination of urinary SCDCA may be clinically useful as a liver function test in chronic liver disease.

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