Enzymic Measurement of Primary Bile Acids and the Primary Bile Acid Ratio in Serum with the IL-Multistat III Fluorescence Light-Scattering Centrifugal Analyzer

A. PAPANASTASIOU-DIAMANDI, E. P. DIAMANDIS and S. J. SOLDIN

Department of Biochemistry, Hospital for Sick Children and Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario Canada M5G 1X8

Enzymic fluorimetric methods are described for the determination of primary bile acids and of chenodeoxycholic acid (CDC) and cholic acid (C) in serum. Bile acids are extracted from 0.3 mL of serum in a simple 5-min step with use of Sep-Pak C_{18} cartridges. Total primary bile acids are measured by an equilibrium technique after reaction with β-NAD^+ in the presence of 7α-hydroxysteroid dehydrogenase. Chenodeoxycholic acid (and its conjugates) is measured by a reaction-rate technique employing the same reaction as above but under different experimental conditions. A small contribution of cholic acid (and its conjugates) to the reaction rate is eliminated by simple calculations. Cholic acid is calculated by difference of the two determinations. In both assays NADH fluorescence is measured with the Multistat centrifugal analyzer. Absolute recovery of bile acids from serum was about 87%. Day-to-day standard deviations for CDC and C were 1.6 and 2.0 μmol/L at serum concentrations of 22.1 and 24.1 μmol/L respectively. Comparison data with a cholylglycine RIA procedure gave the following correlation coefficients (x = RIA, y = proposed method): r = 0.980 (RIA *vs* total primary bile acids), r = 0.918 (RIA *vs* CDC) and r = 0.989 (RIA *vs* C). The methods described appear more practical for use on a routine basis than methods in the literature for the calculation of the primary bile acid ratio.

KEY WORDS: enzymic analysis, fluorescence, bile acids, liver diseases, centrifugal analyzers, primary bile acid ratio, cholestasis

The measurement of total bile acids in serum is a sensitive test of liver function (1, 2). It is accepted that elevation of total bile acids in serum occurs in both hepatocellular injury and cholestatic syndromes. It was recognized early that certain bile acid patterns in serum exist in various liver diseases, and that the measurement of individual bile acids offers additional information for differential diagnosis of liver diseases. The most useful diagnostic information can be obtained by measuring the primary bile acid ratio. It has been shown that a cholate/chenodeoxycholate ratio of greater than one is an indicator of intra- or extrahepatic cholestasis, while a ratio of less than one indicates hepatocellular injury (1, 2). The primary bile acid ratio is also useful in revealing the extent of liver injury and thus it can be used as a prognostically significant test (1). Recently, the diagnostic value of the primary bile acid ratio in various hepatobiliary diseases was confirmed by use of discriminant function analysis (3).

The methods for measuring bile acids in serum can be divided into research and routine procedures. Gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) are probably the most powerful techniques for measurement and characterization of individual bile acids. They are used primarily for research purposes, being time-consuming due to the various separation and derivatization steps. GC-MS methods for serum bile acids have recently been reviewed (4). High-performance liquid chromatographic methods (HPLC) efficiently separate individual bile acids from each other but suffer the serious drawback of being very slow (one to two samples per hour), and need prior extraction of bile acids from serum; detection due to the low molar absorptivity of the bile acids is difficult (5) and is usually accomplished by applying a specific reaction (6). GC, GC-MS and HPLC methods are capable of measuring both individual bile acids and the primary bile acid ratio.

Radioimmunoassays (RIA) for various bile acids in serum have already been described and are commercially available (7–9). These methods are very sensitive (detection limits of 0.1–0.2 μmol/L) and simple to perform, as they are applied directly to serum. RIA procedures measure only a fraction of the total bile acids present in serum and an increase in this fraction is assumed to represent an increase in the total bile acid concentration. Most RIA procedures lack specificity due to cross reactivities which exist for bile acids other than the bile acid of primary interest. Standardization of procedures is therefore difficult (7–9) and reagent cost is high. RIA measurements performed using any of the available commercial kits are not able to measure the primary bile acid ratio.

Enzymic methods for the measurement of bile acids in serum are based on the oxidation of bile acids by β-NAD^+ in the presence of the enzyme 3α- or 7α-hydroxysteroid dehydrogenase (3α-HSD, 7α-HSD) which are commercially available. The liberated NADH can be measured by UV absorption (10), fluorescence (11) or bioluminescence (12) techniques. Total (3α-HSD) or primary (7α-HSD) bile acids can be measured. The measurement of total bile acids in serum is a test which has already been applied on the IL-Multistat III Fluorescence/Light Scatter Microcentrifugal Analyzer (MCA) and this method has recently been modified (13) to improve performance.

Correspondence: Dr. S. J. Soldin, Department of Biochemistry, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8.

Manuscript received November 25, 1983; accepted for publication February 1, 1984.

The primary bile acids, cholic (C) and chenodeoxycholic (CDC) acid, are present in serum as free acids or as glycine (GC, GCDC) or taurine (TC, TCDC) conjugates. The primary bile acid ratio is the concentration ratio of cholic acid (and its conjugates) to chenodeoxycholic acid (and its conjugates), frequently referred to as trihydroxy/dihydroxy concentration ratio. In our previous studies on the kinetics of reaction of primary bile acids with β-NAD$^+$ in the presence of 7α-HSD (14, 15), we found that CDC, GCDC and TCDC have identical reaction rates. The same was true for C, GC, and TC. The reaction rate is about eight times faster for CDC and its conjugates than for C and its conjugates. On the basis of these observations, we devised a method for the analysis of CDC and C mixtures by performing two separate determinations. In both, NADH fluorescence was monitored and the enzyme present was 7α-HSD. In the first determination total primary bile acids are measured by an equilibrium (end-point) technique. In the second determination total CDC (CDC, GCDC, TCDC) is measured in the presence of C and its conjugates by a reaction-rate technique; the small contribution of C and its conjugates in the reaction was eliminated by mathematical treatment of the results. Total cholic acid (C, GC, TC) is measured by the difference between the two determinations.

In this paper, our original manual method (15) is greatly improved. Only 0.3 mL of serum is required. Both acids are conveniently isolated from serum by use of commercially available reverse phase octadecylsilane-bonded silica cartridges in a 5-min step, and are quantified employing the Multistat III Fluorescence/Light Scatter Microcentrifugal Analyzer. The proposed method is accurate, precise and sensitive, and is one of the simplest and fastest methods available for the estimation of the primary bile acid ratio in serum, for diagnostic and prognostic purposes.

Materials and methods

Apparatus

Multistat III Fluorescence/Light Scatter Microcentrifugal Analyzer and Microcentrifugal Loader (nos. 2095 and 818) was manufactured by Instrumentation Laboratory, Spokane, WA 99207. This analytical system has been previously described (16). Sep-Pak C_{18} cartridges (9 mm × 10 mm i.d.) were purchased from Waters Associates, Inc., Milford, MA 01757.

Reagents

All solutions were prepared with reagent-grade materials and deionized-distilled water.

Solution 1

Glycine buffer, 0.1 mol/L, pH 9.5, was prepared by dissolving 7.51 g glycine (Sigma Chemical Company, St. Louis, MO 63178) in about 900 mL of water, adjusting the pH to 9.5 with 6 mol/L NaOH and diluting to 1 L. This solution was stable at room temperature.

Solution II

Glycine-hydrazine sulfate buffer, 1 mol/L, pH 9.5, was prepared by dissolving 7.51 g of glycine and 13.0 g of hydrazine sulfate (Sigma) in about 80 mL water, adjusting the pH to 9.5 with 18 mol/L NaOH and diluting to 100 mL. This solution also was stable at room temperature.

Solution III

Phosphate buffer, 0.1 mol/L, pH 7.5, was prepared by dissolving 13 g of $NaH_2PO_4.H_2O$ (Fisher Scientific Company, Fair Lawn, NJ 07410) in about 900 mL of water, adjusting the pH to 7.5 with 6 mol/L NaOH and diluting to 1 L. This solution was stable at room temperature.

Solution IV

Tris buffer, 0.02 mol/L containing 2 mmol/L EDTA, pH 7.2, was prepared by dissolving 0.484 g of Tris (hydroxymethyl) aminomethane and 0.149 g of Na_2-EDTA·$2H_2O$ in about 150 mL of water, adjusting the pH to 7.2 with 0.5 mol/L HCl and diluting to 200 mL. This solution was stored in a refrigerator when not in use.

Absolute methanol (Anachemia, Mississauga, Canada).

β-NAD$^+$ (Sigma, grade VII, sodium salt). A 2.5×10^{-2} mol/L solution was prepared by dissolving 0.178 g in 10.0 mL of water and kept frozen.

Bile acid standards

Stock (1 mmol/L) solutions of cholic acid and chenodeoxycholic acid were prepared by dissolving 39.26 mg of CDC and 40.86 mg of C (Sigma) in a few drops of 1 mol/L NaOH solution and diluting to 100 mL with water. Working standards were prepared by dilution with water. For routine measurements CDC solutions of 10, 30, 60 and 100 μmol/L were used. When kept refrigerated, these are stable for at least one year.

Enzyme solutions

7α-hydroxysteroid dehydrogenase from *E. coli* (E.C.1.1.1.159), with activity of 6.2 U/mg of solids, partially purified, was purchased from Sigma. A 1.0 U/mL enzyme solution in solution IV was prepared. This solution was pipetted in 10 mL glass tubes, in 0.250 mL aliquots (for the end-point method) and 0.050 mL aliquots (for the kinetic method) and frozen. Frozen aliquots are stable for at least two months.

Working reagents

(a) End-point method. Dilute the 0.250 mL enzyme aliquot with 2.5 mL of solution II. These volumes will permit measurement of primary bile acids in 20 samples. The solution is stable for one day if stored in

an ice-bath. (b) Kinetic method. Add to the tube containing the 0.050 mL enzyme aliquot 8.0 ± 0.5 mg of β-NAD^+ and dilute with 5.0 mL of solution I. This solution should be stored in an ice-bath and will allow measurement of CDC concentrations in 40 samples.

Quinine solutions

A stock solution (200 μmol/L of quinine) was prepared by dissolving 0.0802 g of quinine sulfate dihydrate in 100 mL of 0.05 mol/L H_2SO_4, and stored refrigerated in an amber-glass bottle. Working quinine solutions of 0.4 μmol/L and 0.2 μmol/L were prepared every two months by dilution with 0.05 mol/L H_2SO_4.

Method comparisons were performed using the cholylglycine (CG) radioimmunoassay method (Abbott Laboratories, Diagnostic Division, North Chicago, IL 60064). The latter test was performed according to the manufacturer's instructions.

PROCEDURE

(a) Extraction of bile acids

Pipet 0.300 mL of standards or serum samples in 10 mL glass tubes and dilute with 2.0 mL of solution III. Vortex mix. Prepare the Sep-Pak cartridge as follows: wash the cartridge with 5 mL methanol followed by 5 mL of water. Apply the diluted sample or standard slowly to the Sep-Pak with pasteur pipette and allow to drain. Wash the Sep-Pak once with 3 mL of water. Elute the bile acids slowly in 10 mL glass tubes with 2 mL (2 × 1 mL) of methanol. Evaporate the eluent to dryness under a stream of nitrogen at 60°C. Redissolve in 0.300 mL of solution I by thorough vortexing.

(b) Rotor loading

Pipet manually 200 μL of quinine (0.4 μmol/L for the end-point method and 0.2 μmol/L for the kinetic method) in cuvette no. 1 of the disposable rotor and load cuvettes 2-20 by using the loader with the settings shown in Table 1. Load standards followed by serum samples.

(c) Measurement step

All measurements were carried out at 30°C by employing an excitation wavelength of 350 nm and an emission wavelength of greater than 425 nm by using the instrument's filter positioned in slot no. 9. The tape "F/LS Chemistry" is loaded on the instrument and the investigational program no. 24 "Intensity/Time" is used for data reduction. Table 2 indicates the parameters entered from the keyboard for the end-point and kinetic assays.

For the end-point assay, the loaded rotor is first run with the parameters shown in Table 2 under the heading "blank". After the end of this run, the rotor is taken out of the instrument and 10 μL of 2.5 × 10^{-2} mol/L solution of β-NAD^+ is pipetted manually in the outer hole of the rotor, beginning with cuvette no. 2. Then the rotor is rerun with the parameters shown in Table 2 under the heading "final measurement". The kinetic method is completed in one run since serum blanks are not needed.

TABLE 1
Settings of the Multistat III Loader for the End-Point and Kinetic Measurement of Bile Acids in Serum

Loader setting	End-point	Kinetic
2nd reagent	Off	Off
Reagent-diluent	Reagent	Reagent
Sample		
Sample volume	30% (30 μL)	50% (50 μL)
Total volume	90% (90 μL)	99% (99 μL)
Reagent		
Reagent volume	38% (95 μL)	38% (95 μL)
Total volume	40% (100 μL)	40% (100 μL)

(d) Calculations: End-point method

To construct the calibration curve, plot the fluorescence intensity difference, $\Delta F = F_{final} - F_{blank}$ (F_{final} and F_{blank} are the mean fluorescence intensities of a given cuvette) *versus* the CDC concentration in μmol/L of the standards. Calculate the primary bile acid concentration of the unknowns, [PBA], from this calibration curve.

For the kinetic determination of CDC, calculate the fluorescence intensity change with time, $\Delta F\ s^{-1}$ for each cuvette, by regression analysis of the obtained data. To construct the calibration curve, plot $\Delta F\ s^{-1}$ *versus* the CDC concentration of the standards. Calculate the $[CDC]_{uncorr}$ from this calibration curve. The corrected concentrations of CDC and C in the samples are calculated from the formulas (14).

$$[C] = \frac{[PBA] - [CDC]uncorr}{0.88}$$

$$[CDC] = [PBA] - [C]$$

Results

LINEARITY

Calibration curves for the end-point and kinetic assays can be conveniently constructed through use of CDC solutions alone. It was previously shown (14, 15) that CDC, GCDC and TCDC react with identical rates to β-NAD^+ and any of these acids alone is suitable for the calibration of the kinetic method. Both CDC and its conjugates and C and its conjugates have a 7α-OH group and so they can be oxidized by β-NAD^+ in the presence of 7α-HSD. At the end of the reaction, both CDC and C give rise to equivalent amounts of NADH and so CDC solutions alone can be used to calibrate the end-point assay. The fact that the same concentrations of CDC and C give rise to the same amount of NADH when measured by the end-point assay was confirmed experimentally in this study. Fluorescence intensities

TABLE 5
Absolute Recovery of Primary Bile Acids Added to Human Sera

Primary bile acids, μmol/L			
Initially present*	Added (CDC + C)	Recovered	% recovery
8.6	45.4 (22.7±22.7)	40.9	90
	81.8 (40.9±40.9)	72.8	89
0.0	45.4 (22.7±22.7)	38.2	84
	81.8 (40.9±40.9)	71.5	87
21.6	45.4 (22.7±22.7)	41.2	91
	81.8 (40.9±40.9)	73.1	89
1.4	81.8 (54.5±27.3)	69.9	85
	81.8 (27.3±54.5)	74.9	92
0.6	81.8 (61.4±20.4)	63.6	78
	81.8 (20.4±61.4)	67.0	82
0.8	81.8 (65.9±15.9)	69.3	85
	81.8 (15.9±65.9)	75.1	92
			Av. 87.0±4.3

*Mean of duplicate measurements.

RECOVERY

Absolute recovery of aqueous solutions of bile acids from the Sep-Pak was assessed at 30 μmol/L and 100 μmol/L concentrations. Mean recoveries found were 84% for C, 86% for TC, 76% for GC, 91% for GCDC and 75% for CDC. An overall recovery of 83% was observed. Absolute recovery of primary bile acids from serum was estimated by adding CDC and C in six sera and calculating the recovered bile acids measured by the proposed methods, from calibration curves constructed with CDC solutions which were not subjected to Sep-Pak treatment. Absolute recovery studies are presented in Tables 5 and 6. It can be seen that average absolute recovery is 87.0 ± 4.3% for the primary bile acids, 84.7 ± 9.2% for CDC and 92.9 ± 11.3% for C. The small losses of added bile acids during the analytical process are compensated for by subjecting the standards to the same analytical procedure used for the samples.

COMPARISON STUDIES

We analyzed 102 serum samples from pediatric patients for which bile acid analysis was requested. The same samples were also analyzed by a radioimmunoassay procedure (described in the *Materials and Methods*). The correlation between our end-point method (primary bile acids) and the RIA method (Cholylglycine) is shown in Figure 2. As expected, the correlation between methods is good ($r = 0.980$) but the absolute values differ because the two methods measure different fractions of total bile acids. Thirty-six of the analyzed samples had primary bile acid concentrations greater than 15 μmol/L and were analyzed also by the kinetic procedure, to calculate their CDC and C content. The correlation between methods for CDC and C is presented in Figures 3 and 4. Again, as expected, the correlation with RIA is best for C since the RIA method measures primarily the cholylglycine content of the samples.

We also analyzed sera obtained from 15 healthy male and female employees with age between 20 and 40 years. Blood was withdrawn at 8 a.m. after an overnight fast and exactly 2 hours after their lunch. Mean fasting primary bile acids were found to be 5.6 μmol/L with a standard deviation of 1.9 μmol/L while the 2-hour postprandial levels were 6.8 ± 1.9 μmol/L. These data suggest the upper limit of normal in an adult population of 9.4 μmol/L and 10.6 μmol/L for fasting and postprandial conditions respectively. These normal ranges agree reasonably well with results reported previously by using enzymic techniques (2).

Discussion

Older methods for bile acid isolation from serum include extraction with organic solvents. These methods are now obsolete and have been replaced by liquid-solid extraction with use of the resin Amberlite XAD-2 (15). Since the introduction of octadecylsilane bonded-phase packings in cartridges, an increasing number of publications have appeared in the literature in which these cartridges are employed for the initial isolation of steroids and bile acids from biological fluids (4–6, 17–22). The cartridges have the potential advantage over the XAD-2 resins of providing better recoveries. They are very easy to handle and the whole procedure can be performed semiautomatically with specially designed vacuum apparatus which can process up to 60 samples per hour (4, 20).

During our preliminary experimentation with the end-point and kinetic procedures, we noticed that fluorescence intensity measurements taken a short time after the acceleration of the rotor were not reproducible. We found that at least 100 s is needed after the acceleration of the rotor, to obtain stable and reproducible measurements. We thus use at least 100 s delay time in our measurements. In the kinetic method, the change of fluorescence intensity of the sample *versus* time is linear in the 100 s to 430 s time interval (Figure 1), and so the reaction rate ($\Delta F\ s^{-1}$) can be calculated by linear regression analysis of the fluorescence data printed out by the instrument. In the end-point assay, the reaction is completed during the delay time of 300 s and the final fluorescence is taken as the mean of ten measurements performed during a measurement period of one minute. The CV of the ten fluorescence values printed was always in the range of 0.5 to 1.5%.

To provide better precision we performed duplicate analyses in all standards and samples. The final volume of standards or samples available after Sep-Pak treatment is 300 μL, of which only 80 μL is consumed for both the end-point and the kinetic assay. So, with the same standards at least three complete runs can be performed. Also, very concentrated samples can be diluted with glycine buffer and reanalyzed without the need for repeating the Sep-Pak extraction.

In our previous studies on the kinetics of reaction of CDC and C with β-NAD^+ in the presence of 7α-HSD, we found that CDC and its conjugates react at a rate eight

TABLE 2
Multistat III Program Parameters for the End-Point and Kinetic Determination of Bile Acids in Serum

	End-Point		
	Blank	Final Measurement	Kinetic
Program code	24	24	24
Excitation wavelength (nm)	350	350	350
Reference cuvette	1	1	1
Last cuvette	Dependable[a]	Dependable	Dependable
Delay time (s)	100	300	100
Interval time (s)	6	6	30
No. of intervals	10	10	12
Filter	9	9	9
Start mode	0	0	0
With stats?[b]	Yes	Yes	No
Temperature	30°C		30°C

[a]Up to 20.
[b]This parameter is entered after the end of the run.

TABLE 3
Within-Day Precision for Bile Acids in Serum by the End-Point and Kinetic Procedures

Concentration level	Primary bile acids				Cholic acid			Chenodeoxycholic acid		
	1	2	3	4	2	3	4	2	3	4
Mean (μmol/L)	3.8	39.7	69.9	86.5	20.3	35.7	39.8	19.3	34.3	46.7
SD (μmol/L)	1.8	1.6	1.8	2.5	2.0	2.1	2.5	2.5	2.0	1.5
CV, %	47.4	4.0	2.6	2.9	9.8	6.0	6.3	13.0	5.8	3.2
n	10	10	10	10	10	10	10	10	10	10

TABLE 4
Day-to-Day Precision for Bile Acids in Serum by the End-Point and Kinetic Procedures

Concentration level	Primary bile acids			Cholic acid		Chenodeoxycholic acid	
	1	2	3	2	3	2	3
Mean (μmol/L)	6.5	45.4	86.4	24.1	45.9	22.1	42.0
SD (μmol/L)	1.6	1.3	3.5	2.0	4.3	1.6	3.6
CV, %	24.6	2.9	4.0	8.3	9.4	7.2	8.6
n[a]	16	16	16	16	16	16	16

[a]Over a period of 23 days.

for the blank determinations in the end-point assay were typically in the range of 500–700 arbitrary fluorescence units. The maximum fluorescence reading on the instrument is about 3500 arbitrary fluorescence units. Calibration curve equations for the end-point method (ΔF *versus* concentration of CDC standards in μmol/L) were of the form $y = 13.5x + 50$ with r typically greater than 0.999 and linearity up to 100 μmol/L. Calibration curves for the kinetic method, ΔF s^{-1} *versus* [CDC], were of the form $y = 0.04x$ with almost zero intercept, r greater than 0.999 and linearity up to 60 μmol/L.

PRECISION

Within-day and day-to-day precision were checked by analyzing pooled serum samples. For each individual measurement the whole analytical procedure was performed including the initial extraction step with the Sep-Pak. Precision data are summarized in Tables 3 and 4. Coefficients of variation for the end-point method range between 2.5 and 4% and for the kinetic method between 3.2 and 13%. Similar or inferior precision data have been reported for the radioimmunoassay techniques (7).

TABLE 6
Absolute Recovery of CDC and C Added to Human Sera

Primary bile acids, μmol/L							
Initially present		Added		Recovered		% recovery	
CDC	C	CDC	C	CDC	C	CDC	C
4.3	4.3	22.7	22.7	16.0	24.9	70	110
		40.9	40.9	33.5	39.3	82	96
0.0	0.0	22.7	22.7	21.3	16.9	94	74
		40.9	40.9	33.6	37.9	82	93
3.9	17.7	22.7	22.7	21.1	20.1	93	88
		40.9	40.9	34.6	38.5	85	94
0.6	0.8	54.5	27.3	44.1	25.8	81	94
		27.3	54.5	25.0	49.9	92	92
0.3	0.3	61.4	20.4	45.7	17.9	74	88
		20.4	61.4	17.1	49.9	84	81
0.4	0.4	65.9	15.9	50.8	18.5	77	116
		15.9	65.9	16.2	58.9	102	89
						Av. 84.7±9.2	92.9±11.3

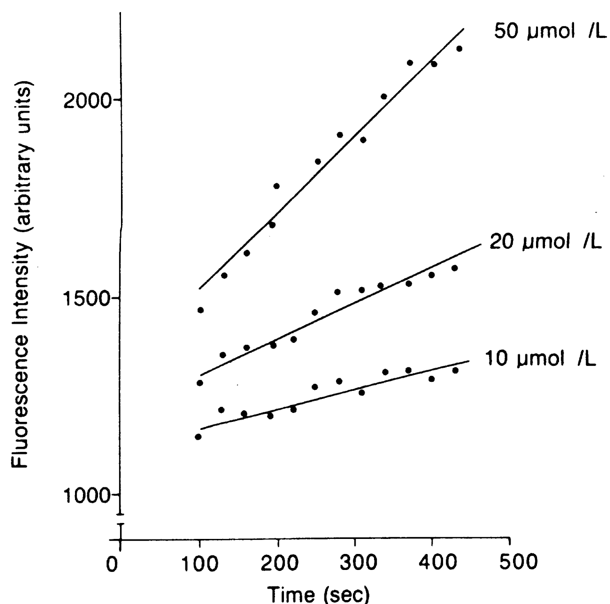

Figure 1 — Change of fluorescence intensity with time for the reaction of CDC with β-NAD$^+$ in the presence of the enzyme 7α-hydroxysteroid dehydrogenase. Concentrations of CDC in μmol/L are shown. Other conditions as under procedure.

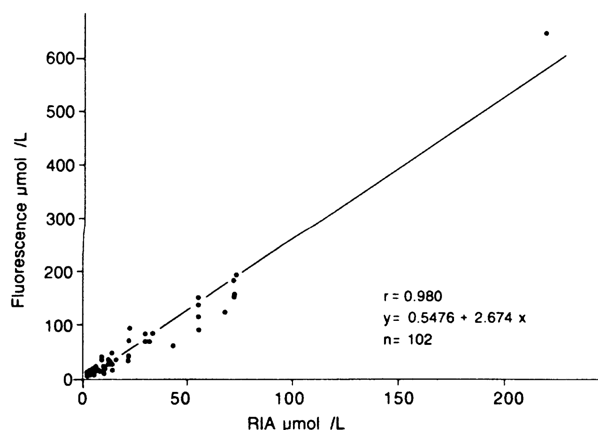

Figure 2 — Correlation between the proposed method for primary bile acids and the cholylglycine radioimmunoassay procedure.

times faster than C and its conjugates. The small contribution to the reaction rate from C and its conjugates can be successfully removed by using the simple equation given to interpret the results as described in detail elsewhere (14).

The kinetic method for CDC is not very sensitive at concentrations of CDC below 10 μmol/L. This is only a minor limitation as the C/CDC ratio is found useful in disease states where bile acids are usually moderately to highly elevated. Samples which contain less than 20 μmol/L of the primary bile acids (as measured by the end-point method which is performed first) do not need to be subjected to the kinetic method assay for the calculation of the C/CDC ratio. The elevation of primary bile acids in serum has the same clinical significance as the elevation of total bile acids as measured by use of 3α-HSD, because in disease states the elevation of total bile acids is almost exclusively due to elevation of primary bile acids (1). The C/CDC ratio can be estimated by the proposed method in samples containing primary bile acids less than 20 μmol/L if 1.5 mL of sample is applied to the Sep-Pak and the procedure described is followed afterwards (5-fold preconcentration).

An advantage of the methods described here over existing enzymic or radioimmunoassay methods is the significantly lower cost of reagents per test and the prolonged stability of the reagents used. The most labile reagent is the enzyme which can be aliquoted and kept frozen in solution for at least two months.

Very occasionally, a sample subjected for analysis exhibited a very high blank value (e.g., over 1200 arbitrary fluorescence units by the end-point method) which makes the measurement difficult or impossible.

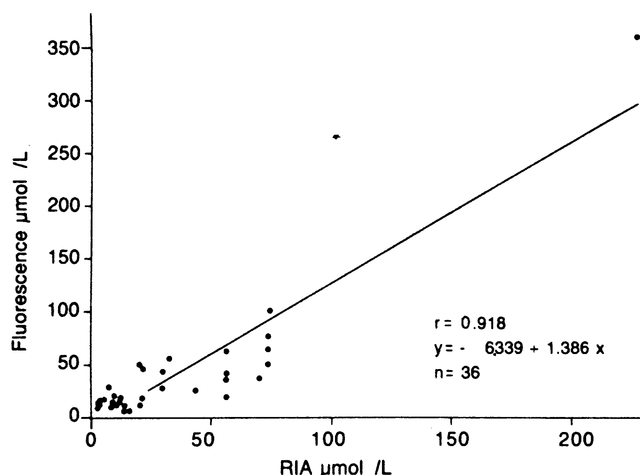

Figure 3 — Correlation between the proposed method for chenodeoxycholic acid and the cholyglycine radioimmunoassay procedure.

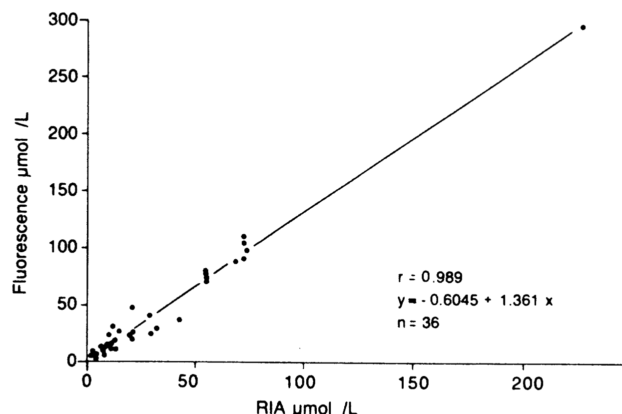

Figure 4 — Correlation between the proposed method for cholic acid and cholylglycine radioimmunoassay procedure.

Presumably, this effect is due to drugs or drug metabolites in the serum. These samples can either be diluted before analysis or a new sample requested when the patient is not on medication.

Acknowledgements

We wish to thank Dr. D. M. Goldberg for providing financial support to one of us (APD) and for advice and encouragement, and Dr. G. Ellis who provided us with serum samples previously assayed for bile acids by the RIA procedure.

References

1. Carey JB Jr. Bile salt metabolism in man. In: Nair PB and Kritchevsky D Eds. *The Bile Acids*, Vol. 2. Pp. 55–82. New York: Plenum Press, 1973.
2. Javitt NB. Bile acids and hepatobiliary disease. In: Schiff L and Schiff ER Eds. *Diseases of the Liver*, 5th ed. Pp. 119–50. Philadelphia: Lippincott, 1982.
3. Linnet K, Andersen JR. Differential diagnostic value in hepatobiliary disease of serum conjugated bile acid concentrations and some routine liver tests assessed by discriminant analysis. *Clin Chim Acta* 1983; **127**: 217–28.
4. Setchell KDR, Matsui A. Serum bile acid analysis. *Clin Chim Acta* 1983; **127**: 1–17.
5. Kamada S, Maeda M, Tsuji A, Umezawa Y, Kurahashi T. Separation and determination of bile acids by high-performance liquid chromatography using immobilized 3α-hydroxysteroid dehydrogenase and an electrochemical detector. *J Chromatogr* 1982; **239**: 773–83.
6. Linnet K. A high-pressure liquid chromatographic-enzymatic assay for glycine and taurine conjugates of cholic, chenodeoxycholic and deoxycholic acid in serum. *Scand J Clin Lab Invest* 1982; **42**: 455–60.
7. Roda A, Roda E, Aldini R, *et al.* Results with six "kit" radioimmunoassays for primary bile acids in human serum intercompared. *Clin Chem* 1980; **26**: 1677–82.
8. Miller P, Weiss S, Cornell M, Duckery J. Specific ^{125}I-radioimmunoassay for cholylglycine, a bile acid, in serum. *Clin Chem* 1981; **27**: 1698–703.
9. Wildgrube HJ, Stockhausen H, Metz P, Mauritz A, Mahdawi R. Radioimmunoassay of bile acids in tissue, bile and urine. *Clin Chem* 1983; **29**: 494–8.
10. Mashige F, Tanaka N, Maki A, Kamei S, Yamanaka M. Direct spectrophotometry of total bile acids in serum. *Clin Chem* 1981; **27**: 1352–6.
11. Siskos PA, Cahill PT, Javitt NB. Serum bile acid analysis: a rapid, direct enzymatic method using dual-beam spectrophotofluorimetry. *J Lipid Res* 1977; **18**: 666–71.
12. Roda A, Kricka LA, DeLuca M, Hofmann AF. Bioluminescence measurement of primary bile acids using immobilized 7α-hydroxysteroid dehydrogenase: application to serum bile acids. *J Lipid Res* 1982; **23**: 1354–61.
13. Hanson Q, Freier EF. Effect of protein on the determination of total bile acids in serum. *Clin Chem* 1983; **29**: 171–5.
14. Papanastasiou-Diamandi A, Siskos PA, Hadjiioannou TP. Enzymatic fluorimetric determination of cholic acid and chenodeoxycholic acid in aqueous solutions and serum using a differential kinetic method. Accepted for publication in *Anal Chim Acta* 1984; **157**: 125–33.
15. Papanastasiou-Diamandi A, Siskos PA, Hadjiioannou TP. Enzymatic fluorimetric determination of total, primary and individual bile acids in serum. Accepted for publication in *Anal Chim Acta*.
16. Hills LP, Tiffany TO. Comparison of turbidimetric and light scattering measurements of immunoglobulins by use of a centrifugal analyzer with absorbance and fluorescence/light scattering optics. *Clin Chem* 1980; **26**: 1459–66.
17. Axelson M, Sahlberg BL. Solid extraction of steroid conjugates from plasma and milk. *Anal Letters* 1981; **14**: 771–82.
18. Shackleton CHL, Whitney JO. Use of Sep-Pak cartridges for urinary steroid extraction. Evaluation of the method for use prior to gas chromatographic analysis. *Clin Chim Acta* 1980; **107**: 231–43.
19. Heikkinen R, Fotsis T, Aldercreutz H. Reversed-phase C_{18} cartridges for extraction of estrogens from urine and plasma. *Clin Chem* 1981; **27**: 1186–9.
20. Setchell KDR, Worthington J. A rapid method for the quantitative extraction of bile acids and their conjugates from serum using commercially available reverse phase octadecylsilane bonded silica cartridges. *Clin Chim Acta* 1982; **125**: 135–44.
21. DeMark BR, Everson GT, Klein PD, Showalter RB, Kern F. A method for the accurate measurement of isotope ratios of chenodeoxycholic and cholic acids in serum. *J Lipid Res* 1982; **23**: 204–10.
22. Smith SM, Mysov M, Setchell KDR. Measurement of conjugated and unconjugated serum bile acid concentration using 3α-hydroxysteroid dehydrogenase. *J Clin Pathol* 1983; **36**: 235–6.