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Direct enzymic fluorimetric method for the determination of individual bile acids in bile

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Summary

We describe a new, simple, fluorimetric enzymic method for the determination of individual bile acids in bile. Bile is diluted 4000-fold with water and 3α- and 7α-hydroxy bile acids are determined by equilibrium methods and chenodeoxycholic acid is determined by a differential kinetic method, without any prior separation and preparatory step. The equilibrium methods are based on the reaction of 3α- or 7α-hydroxy bile acids with β-NAD^+ in the presence of the enzyme 3α- or 7α-hydroxysteroid dehydrogenase (HSD). The kinetic method is based upon the reaction of chenodeoxycholic acid with β-NAD^+ in the presence of the enzyme 7α-HSD. All measurements are monitored fluorimetrically. Cholic and deoxycholic acid are calculated by difference. Recovery experiments gave satisfactory results. Gallbladder bile from patients was analysed for the three major bile acids. The proposed method is suitable for clinical use.

Introduction

The concentration of bile acids in bile is more than 1000-fold greater than in serum. Therefore, the analytical procedures for the determination of bile acids in bile are simpler compared with those for serum. The biliary bile acids have generally been analysed by thin-layer chromatography (TLC) [1], gas-liquid chromatography (GLC) [2] and high-performance liquid chromatography (HPLC) [3,4]. Analysis by TLC is time-consuming and requires an enzymic or chemical method for quantification after separation of individual bile acids. On the other hand analysis by GLC has the laborious steps of hydrolysis and derivatisation prior to the injection of the sample into the chromatograph. HPLC is relatively simple, but the equipment is expensive and complicated to use.

Macdonald and coworkers [5] described an enzymic method for the determination

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of the three main bile acids, cholic (C), chenodeoxycholic (CDC) and deoxycholic acid (DC) in bile, by determining the 3α-, 7α- and 12α-hydroxy bile acids by three equilibrium procedures, using three enzymes: 3α-, 7α- and 12α-hydroxysteroid dehydrogenase (HSD). However, this method cannot be used on a routine basis, because 12α-HSD is not commercially available.

Recently, we developed a new enzymic fluorimetric method for the determination of C, CDC and DC in serum without any prior separation [6]. In this report we describe a method for direct application in bile. Bile is diluted 4000-fold and the following determinations are carried out: (a) equilibrium determination of 3α-hydroxy bile acids using 3α-HSD; (b) equilibrium determination of 7α-hydroxy bile acids using 7α-HSD; and (c) kinetic detemination of chenodeoxycholic acid using 7α-HSD.

The method is suitable for the analysis of biliary bile acids in many pathological conditions, especially for the investigation of pathogenesis of gallstone formation, and in small clinical laboratories where filter fluorometers or simple fluorescence spectrophotometers are available.

Materials and methods

Apparatus

A double-beam fluorescence spectrophotometer (Model 512, Perkin Elmer, Norwalk USA) with a 150-W xenon lamp was used. All measurements were performed using standard rectangular cells of 1000 cm path-length and under constant stirring with a magnetic stirrer adapted to the sample compartment. A constant temperature of 25.0 ± 0.1°C in the sample cell was maintained by using a water bath (Model ST, Sargent, Chicago, IL, USA). The fluorometric signals were recorded on a Sargent-Welch XKR potentiometric recorder.

The following instrumental settings were used for analysis. Ratio mode, dynode voltage 750 V; excitation wavelength 350 nm; excitation slit 20 nm; emission wavelength 455 nm; emission slit 20 nm. The sensitivity scale factor used to measure fluorescence intensity was at the X10 position for the kinetic determination and X3 position for the equilibrium determinations.

Reagents

All solutions were prepared in doubly distilled deionised water from reagent-grade materials.

3α-HSD (EC 1.1.1.50) (0.50 U/mg; Worthington Biochemicals Corp., Freehold, NJ 07728, USA). It was supplied as powder obtained from *P. Testosteroni.* An enzyme solution of 0.25 U/ml was prepared by dissolving 5.0 mg of the enzyme in 10.0 ml of 0.02 mol/l 'Tris' buffer of pH 7.2, containing 2 mmol/l EDTA. This enzyme solution was stored at −20°C and was stable for at least 1 week.

7α-HSD (EC 1.1.1.159) (0.50 U/mg; Worthington). It was supplied as powder obtained from *E. coli.* Enzyme solutions of 3.0 U/ml and 0.125 U/ml were prepared and stored as in the case of 3α-HSD solution.

β-NAD⁺. A 0.0100 mol/l solution was prepared by dissolving 0.717 g of β-NAD^+ (grade A; Calbiochem, San Diego, CA 92112, USA) in 10.00 ml of water. This solution was kept in a refrigerator and is stable for at least 2 weeks.

Glycine buffer, 0.10 mol/l, pH 9.5, containing EDTA (5 mmol/l) was prepared by dissolving 1.8 g of glycine and 0.465 g of $Na_2EDTA \cdot 2\ H_2O$ in about 200 ml of water, adjusting the pH to 9.5 with NaOH solution and diluting to 250 ml.

Glycine (1.0 mol/l)-hydrazine (0.40 mol/l) buffer of pH 9.5 was prepared by dissolving 18.8 g glycine, 13.0 g hydrazine sulfate and 0.5 g $Na_2EDTA \cdot 2\ H_2O$ in about 200 ml water, adjusting the pH to 9.5 with 5 mol/l NaOH solution and diluting to 250 ml.

Bile acid solutions. Bile acids were purchased from Calbiochem. Stock solutions of bile acids (1 mmol/l) were prepared by dissolving appropriate amounts of the sodium salts of bile acids in water. For the preparation of calibration curves, aqueous solutions of chenodeoxycholic acid, 10–80 μmol/l were used in all cases.

Samples

Human bile was obtained from gallbladder during surgery. The samples were stored at −20°C until analysis.

Bile samples were centrifuged. 25 μl of the supernatant were diluted to 100 ml with water (solution A) and the following determinations were carried out:

(a) Equilibrium determination of 3α-hydroxy bile acids (TBA). Transfer into the cuvette 2.00 ml of glycine-hydrazine buffer, 0.200 ml of standard solution or solution A, 0.100 ml of 3α-HSD solution and start the stirrer. After the fluorescence signal is stabilised (~ 1 min), inject 0.100 ml of β-NAD^+ solution and record the fluorescence intensity until its value reaches the maximum. Measure the fluorescence change (ΔF) from the recorded curves. Construct the calibration curve by plotting ΔF values vs. concentration of standards in μmol/l. The results for the unknown bile samples must be multiplied by the dilution factor (×4000).

(b) Equilibrium determination of 7α-hydroxy bile acids (PBA). As in the procedure (a), except that 7α-HSD solution (3.0 U/ml) is used instead of 3α-HSD. Prepare calibration curve as above.

(c) Kinetic determination of chenodeoxycholic acid (CDC). Transfer into the cuvette 2.00 ml of glycine buffer (0.10 mol/l, pH 9.5), 0.200 ml of standard solution or solution A, 0.100 ml of 7α-HSD solution (0.125 U/ml) and start the stirrer. After the fluorescence signal is stabilised inject 0.100 ml of β-NAD^+ solution and record the fluorescence intensity change for about 3 min. Calculate the initial reaction rate in ΔF/min from the recorded curves (slope technique). Construct the calibration curves by plotting ΔF/min values for standards-vs concentrations in μmol/l. Multiply by dilution factor after the calculation of CDC concentration as described below.

Calculations

The unknown concentrations of cholic acid, chenodeoxycholic acid and deoxycholic acid can be estimated by solving the following equations:

$$[\text{TBA}] = [\text{C}] + [\text{CDC}] + [\text{DC}] \quad (1)$$

$$[\text{PBA}] = [\text{C}] + [\text{CDC}] \quad (2)$$

$$[\text{CDC}]_{\text{uncor}} = R[\text{C}] + [\text{CDC}] \quad (3)$$

where [TBA] (total bile acids) is the concentration of 3α-hydroxy bile acids measured by the procedure (a), [PBA] (primary bile acids) is the concentration of 7α-hydroxy bile acids measured by the procedure (b) and $[\text{CDC}]_{\text{uncor}}$ is the uncorrected concentration of chenodeoxycholic acid measured by the procedure (c) and includes the contribution of cholic acid to the total reaction rate, and R is the ratio of proportionality constants of calibration equations for the kinetic determination of C and CDC (in the present case $R = 0.035/0.290 = 0.121$, that is CDC reacts with β-NAD^+ in the presence of 7α-HSD 8.3 times faster than C. R is a constant under the specified conditions of the assay and there is no need for its determination by the individual user of the method [6].

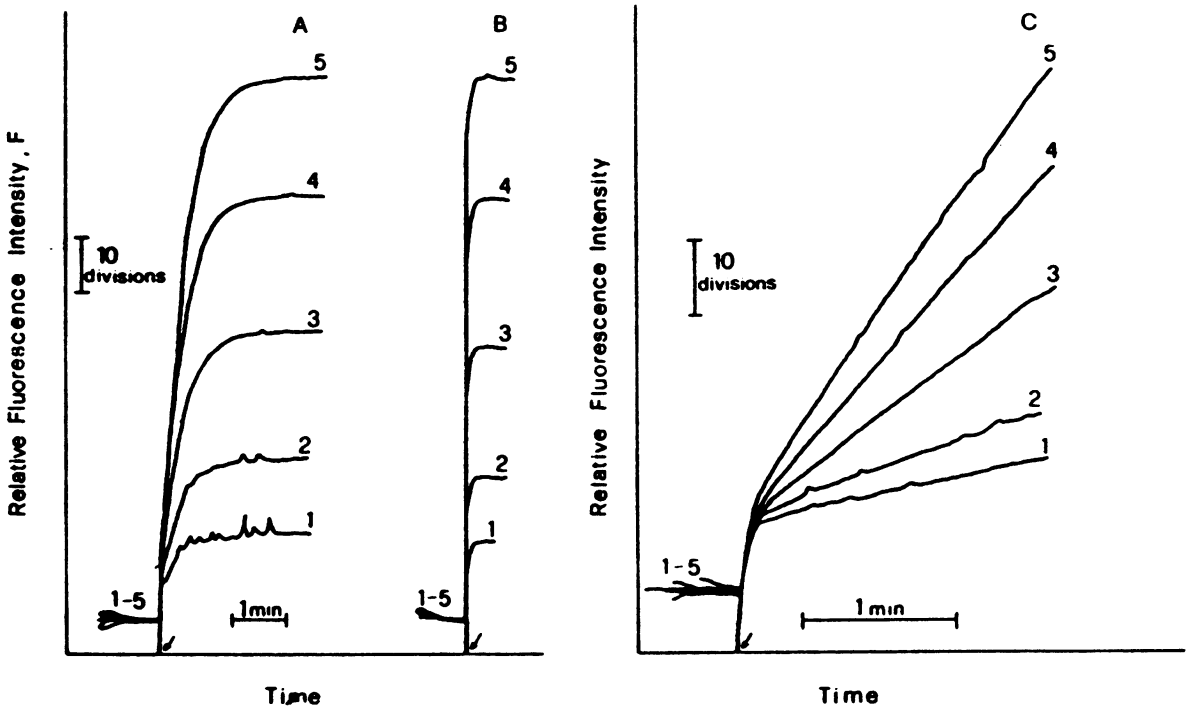

Fig. 1. Typical recording curves of relative fluorescence intensity vs. time taken with aqueous chenodeoxycholic acid solutions. (A) Equilibrium procedure for TBA; (B) equilibrium procedure for PBA (C); differential kinetic method for CDC. Initial concentrations of CDC: (1), 10 μmol/l; (2), 20 μmol/l; (3), 40 μmol/l; (4), 60 μmol/l; and (5), 80 μmol/l. Other conditions as described in 'Methods and materials'. At the time indicated by arrows, the reaction was started by injecting the starting reagent (β-NAD^+ solutions, see 'Reagents').

TABLE I

Reproducibility of the methods for the determination of bile acids in human bile ($n = 8$)

Bile acid	Mean value (mmol/l)	Coefficient of variation (%)
TBA	109.3	1.9
PBA	87.2	2.3
CDC	70.8	4.8
C	16.4	17.1
DC	22.1	13.1

Results

Fig. 1 shows recorded curves for the determination of 3α- and 7α-hydroxy bile acids and chenodeoxycholic acid. The calibration curves for the three determinations are linear up to 80 μmol/l.

A pooled bile sample was analysed by the proposed procedure after several dilutions in the range 2000–5000-fold. The results showed a linear correlation in the above range. A dilution factor of 4000 was chosen for bile samples, so as to obtain adequate sensitivity and to eliminate the interference of other constituents of bile such as proteins, bilirubin etc., which interfere with the determination of bile acids in serum.

TABLE II

Analytical recoveries of cholic acid and chenodeoxycholic acid added to three different pool bile samples

Originally present (mmol/l)	Compound added	Added (mmol/l)	Recovered * (mmol/l)	Recovery of added amount (%)
59.8 **	GC	56.7	67.9	120
36.3 **	TC	66.6	64.6	97
	GC	53.8	49.0	91
	C	107.7	99.2	92
35.4 **	GC	49.2	47.7	97
	TC	51.7	41.0	79
57.4 ***	CDC	59.9	60.8	102
39.1 ***	GCDC	53.6	41.8	78
	CDC	51.2	54.4	106
59.4 ***	GCDC	50.9	55.2	108
	TCDC	49.6	58.9	119

* Mean values of two determinations.

** Cholic acid.

*** Chenodeoxycholic acid.

TABLE III

Bile acid analysis of gallbladder biles from patients [a]

Patient's sample	[TBA] (mmol/l)	[PBA] (mmol/l)	[CDC] (mmol/l)	[C] (mmol/l)	[DC] (mmol/l)
1 [b]	18.1	15.6	8.26	7.34	2.50
2 [b]	202	144	89.6	54.4	58.0
3 [b]	135	116	92.1	23.9	19.0
4 [b]	117	90.3	72.0	18.3	26.7
5 [b]	44.8	22.2	10.4	11.8	22.6
6 [b]	97.1	75.0	54.6	20.4	22.1
7 [b]	51.8	47.5	25.9	21.6	4.30
8 [b]	68.7	52.3	27.0	25.3	16.4
9 [c]	180	157	98.1	58.9	23.0
10 [d]	168	155	80.8	74.2	13.0
11 [d]	73.4	64.7	49.7	15.0	8.70
12 [d]	201	168	123	45.4	33.0

[a] Mean of two determinations.
[b] Cholelithiasis.
[c] Cholesterinosis.
[d] Cancer of the head of the pancreas.

The precision of the method is presented in Table I.

The accuracy of the method was tested with recovery experiments by adding bile acids to bile samples. The average recovery of total bile acids was 94.9% (range 85–105%), of primary bile acids 91.5% (range 84–99%), of chenodeoxycholic acid 102.4% (range 78–119%) and of cholic acid 96.0% range (79–120%). Analytical recoveries for chenodeoxycholic acid and cholic acid are shown in Table II.

The proposed method was applied to the determination of bile acids in gallbladder bile samples from patients with cholelithiasis and other diseases. The results are shown in Table III.

The mean values for patients with cholelithiasis were (91.8 ± 59.1) mmol/l, (70.4 ± 44.9) mmol/l, (47.5 ± 34.2) mmol/l, (22.9 ± 14.1) mmol/l and (21.4 ± 17.1) mmol/l for 3α-hydroxy bile acids, 7α-hydroxy bile acids, chenodeoxycholic acid, cholic acid and deoxycholic acid, respectively. Values of (135.2 ± 64.6) mmol/l and (87.9 ± 59.7) mmol/l are reported for total bile acids for normal and abnormal bile, respectively [7].

Discussion

Bile acids are present in bile in large concentrations and therefore special sensitivity of the methods for the determination of biliary bile acids is not required. Some of the constituents which interfere with the determination of bile acids in serum (i.e. bilirubin, proteins, and lipids [7] are present in high concentrations in bile. In the method described, we eliminated the interference of the above substances

only by dilution of bile samples and measuring the bile acids using fluorescence methodology.

The relatively poor precision for the determination of cholic and deoxycholic acid (Table I) is due to the indirect method of calculation of their concentration.

The proposed method does not discriminate between free bile acids and their conjugates, as do all the enzymic methods. Furthermore, sulphated bile acids are not measured. No studies were carried out with lithocholic acid because it exists only in traces in bile.

The major merits of the present method for the three individual bile acids are the elimination of the preparation of the sample, the simplicity and the low cost. Only two enzymes are used. The method does not require expensive instruments as does high-performance liquid chromatography and radioimmunoassays, and can be used in small clinical chemistry laboratories. Twenty bile samples can be analysed for five classes of bile acids in a working day. It will be useful in the analysis of biliary bile acids in many pathological conditions, especially for the investigation of the pathogenesis of gallstone formation.

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