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A fluorimetric and enzymatic method for the estimation of serum total bile acids

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SYNOPSIS The enzymatic technique using 3α-hydroxysteroid dehydrogenase for determining bile acids in blood has been modified by measuring the reduced nicotinamide adenine dinucleotide fluorimetrically. The increased sensitivity attained enables the concentration of total bile acids in serum to be estimated using 3 ml for normal subjects and 1 ml for jaundiced patients. The range of normal values in serum was found to be 0-4·7 μmol/litre for males and 1·0-8·2 μmol/litre for females.

In the assessment of liver function most substances metabolized by the liver can be measured easily in plasma, except for bile acids, and a reasonably rapid and specific method for their investigation is therefore needed. All the methods previously available have disadvantages: gas liquid chromatography requires lengthy chemical treatment of the sample before analysis (Sandberg, Sjövall, Sjövall, and Turner, 1965) whilst fluorimetry (in concentrated sulphuric acid) depends for its specificity on an initial prolonged extraction and purification process (Panveliwalla, Lewis, Tabaqchali, and Wootton, 1970).

Marcus and Talalay (1956) described an NAD-linked 3α-hydroxysteroid dehydrogenase obtained from *Pseudomonas testosteroni*: this enzyme can be used for the quantitative assay of androsterone and of other androgens with a 3α-hydroxyl substituent (Hurlock and Talalay, 1957). The bile acids (cholic acid, deoxycholic acid, chenodeoxycholic acid) are also 3α-hydroxysteroids and will react with 3α-hydroxysteroid dehydrogenase. The reaction may be represented as

$$\text{Steroid alcohol} + NAD^+ \rightleftharpoons \text{Steroid ketone} + NADH + H^+.$$

Completion of this reaction to the right can be ensured by the use of hydrazine as a ketone trapping agent. Iwata and Yamasaki (1964) used this reaction for the determination of bile acids in blood, and their technique was adapted by Turnberg and Anthony-Mote (1969) for the determination of bile acids in bile. These authors measured NADH spectrophotometrically at 340 nm: Iwata and Yamasaki (1964) found the lowest practical limit of detection to be 5 μg (approximately 0·01 μmol) in the final reaction mixture (3 ml) which means that at least 20 ml serum is needed if values for normal sera are to be estimated. By the use of fluorimetry for the determination of NADH (Lowry, Roberts, and Kapphahn, 1957) it is possible to measure 0·25 μg (approximately 0·0005 μmol) of bile acid in the final reaction mixture (2·05 ml).

Using the specific enzyme reaction, the initial purification need not be so exhaustive. In the method to be described the procedure of Frosch and Wagener (1967) is used: in this, after extraction of serum with hot ethanol, neutral lipids are almost completely removed by distribution in alkaline aqueous alcohol and ether-heptane. By combining the enzymatic conversion of bile acids with the fluorimetric determination of NADH it is possible to measure concentrations of bile acids as low as those found in normal serum.

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Method

APPARATUS

The instrument used was a Locarte fluorimeter, model MK4, with a mercury lamp. An LF1 filter which transmits wavelengths from 254 to 400 nm

was used on the primary (excitation) side, and an LF6 filter which transmits light of 470 nm and greater was used on the secondary (emission) side.

REAGENTS

Sodium pyrophosphate solution (0·1 M)
The solution was adjusted to *p*H 10·2 with 0·1 N NaOH.

Ethanolic sodium pyrophosphate solution
This was prepared daily by adding 5 ml absolute ethanol to 95 ml 0·1 M sodium pyrophosphate solution.

Ethanol
A 50% solution was prepared by diluting absolute ethanol with water and the *p*H was adjusted to 9·5-10·5, using a glass electrode.

Hydrazine hydrate (0·1 M)
This solution was prepared by adding 5 ml hydrazine hydrate (99-100%) to ice-cold water and then slowly adding 1·5 ml 2N H_2SO_4. The volume was made up to 100 ml with distilled water, the *p*H adjusted to 9·5, and the solution stored at 4°C.

β Nicotinamide adenine dinucleotide (NAD)
An approximately 2·4 mM solution of β nicotinamide adenine dinucleotide (obtained from Sigma Chemicals Co.) was prepared immediately before use by dissolving 35 mg in 20 ml aqueous 0·1 M sodium pyrophosphate solution, *p*H 10·2 (sufficient for 20 samples assayed in duplicate).

3α-Hydroxysteroid dehydrogenase (10 mg/ml)
Hydroxysteroid dehydrogenase (obtained from Sigma Chemicals Co.) is a crude preparation of dried cells of *Pseudomonas testosteroni* containing 3α-hydroxysteroid dehydrogenase, 3, 17 β-hydroxysteroid dehydrogenase, and other enzymes active in steroid metabolism. One hundred mg was ground, in a pestle and mortar, with a knife point of alumina in 1 ml ice-cold water to ensure rupture of the bacterial cells. The volume was made up to 10 ml with ice-cold water, and the resulting suspension mixed thoroughly and then centrifuged at 30,000 *g* for 40 min at −0°C. The clear supernatant was used for the bile acid assay. If analysis is carried out infrequently it is advisable to store the enzyme solution in small aliquots at −20°C. It may be kept for four days at 4°C without significant loss of activity.

Pure 3α-hydroxysteroid dehydrogenase (Boyer, Baron, and Talalay, 1965)
This reagent was kindly provided by Dr Paul Talalay (Johns Hopkins University School of Medicine). A 10 mg/ml solution was prepared in the manner described for the Sigma hydroxysteroid dehydrogenase.

Bile acid standard solution
Bile acids were obtained from Maybridge Chemicals Ltd, Launcester, Cornwall, England. With the exception of cholic acid they were found to be 95% pure when examined by thin-layer chromatography. For conjugated bile acids the solvent system reported by Hofmann (1964) was employed, ie, water/*n* propanol/propionic acid/isoamyl acetate (1:2:3:4, v/v), and for unconjugated bile acids the system reported by Gregg (1966), ie, isooctane/isopropyl ether/glacial acetic acid/isopropyl alcohol (2:1:1:1, v/v). The cholic acid preparation contained other bile acids present to a concentration of 25%. As the bile acid present in the highest concentration in normal sera is glycocholic acid, this was selected as standard. A stock solution of sodium glycocholate, 2,050 μmol/litre, was prepared by dissolving 100 mg of the salt in 100 ml 50% aqueous ethanol. From this stock solution working standards, ranging from 10·25 to 205 μmol/litre (5-100 μg/ml), were prepared by diluting the stock solution with 50% aqueous ethanol.

PURIFICATION OF SOLVENTS

All organic solvents (AR grade) used, with the exception of diethyl ether, were redistilled from glass.

Procedure

Ten ml blood was collected from fasting subjects and the sera were stored at −15°C in glass vials.

EXTRACTION

Three ml serum was pipetted dropwise into 15 ml boiling ethanol in a 50-ml conical flask, fitted with a simple air condenser and magnetically stirred. Heating was continued for five minutes. The flask was then allowed to cool to room temperature and the contents were centrifuged for five minutes at 2,000 *g*. The supernatant solution was decanted into a stoppered glass tube, and the precipitate was agitated with 10 ml absolute ethanol and the boiling-stirring procedure repeated. After centrifugation the ethanolic solutions were combined, and evaporated to dryness in a rotary evaporator. The residue was washed into a 60-ml stoppered tube with 2 × 5 ml volumes of 50% ethanol and then extracted with 20 ml ether/heptane (1:1, v/v) by mechanical shaking for 20 minutes (not less than 10 minutes, not more than 25 min, depending on the type of extractor used). The tube was then centrifuged at 1,000 *g* for 10 minutes, after which the upper phase was removed using a pasteur pipette and water pump, and the lower phase evaporated to dryness. The residue was then dissolved in 1 ml 50% ethanol.

ENZYMATIC ASSAY

The following solutions were prepared immediately before use. Reagent A was prepared by adding 10 ml hydrazine hydrate solution to 10 ml NAD and 1 ml enzyme solution contained in a 100 ml volumetric flask, and made up to the mark with ethanolic pyrophosphate. Reagent B was prepared by adding 10 ml hydrazine hydrate solution to 10 ml NAD and diluted to 100 ml with ethanolic pyrophosphate solution.

Tubes were set up, in duplicate, as follows:

NAD blank

To 50 μl 50% ethanol was added 2 ml reagent B. This solution was used to measure the fluorescence of all the reagents without the enzyme preparation.

Reagent blank

To 50 μl 50% ethanol was added 2 ml reagent A. The fluorescence in this tube was derived from all the reagents, including the enzyme preparation. The latter is a large contributor to the blank and it is therefore necessary to keep the amount of enzyme preparation used to a minimum.

Standard test

To 50 μl of each working standard was added 2 ml reagent A. This solution was used to measure fluorescence due to the standard bile acid-enzyme-NAD reaction and the background reagent fluorescence.

Serum test

To 50 μl serum extract was added 2 ml reagent A. This solution was used to measure the fluorescence due to the serum bile acid-enzyme-NAD reaction, and the fluorescence due to serum and reagents.

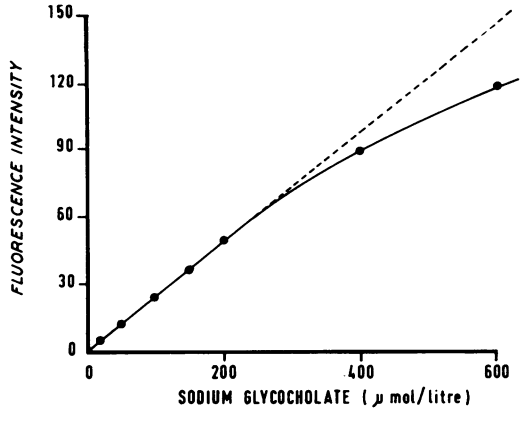

Fig. 1 *Calibration curve for sodium glycocholate showing relationship between intensity of fluorescence (in galvanometer units) and concentration of standard solutions.*

Serum blank

To 50 μl serum extract was added 2 ml reagent B. This solution was used to measure the background fluorescence due to serum and all the reagents except the enzyme preparation.

All the solutions were mixed thoroughly and the tubes placed in water at 37°C for 45 minutes. They were then placed at −20°C for 10 minutes. The tubes were then taken from the refrigerator and allowed to stand at room temperature for 10 minutes so that the solutions were read when they had reached a temperature of 5 to 10°C. It is essential that all readings are made at the same temperature, which should be as low as possible because of the high negative coefficient of fluorescence of NADH (Lowry *et al*, 1957; Greef and Israelstram, 1968).

READING

The galvanometer scale of the fluorimeter was set at zero using the NAD blank, and the sensitivity controls were adjusted so that the top working standard read 50. The fluorescence of the solutions was then read. The reading of the reagent blank was subtracted from the readings of the standards and a calibration curve plotted in the usual way (Fig. 1). The curve was linear up to a bile acid standard concentration of 205 μmol/litre. This is equivalent to a concentration of bile acid in the cuvette of 5 μmol/litre.

CALCULATION OF RESULTS

The concentration of serum bile acids may be read from the standard graph and multiplied by the appropriate factor used in the extraction procedure (in the above description this is 1/3), that is:

$$\text{Concentration of total bile acids in serum } (\mu\text{mol/litre}) = \frac{\text{Serum test} - (\text{reagent blank} + \text{serum blank})}{\text{Standard} - \text{reagent blank}} \times \frac{\text{Concentration standard}}{3}$$

ARTIFICIAL FLUORESCENCE STANDARD

If the fluorimeter is regularly in use for estimations other than bile acids it is convenient to set the sensitivity of the fluorimeter with an artificial standard of fluorescence. The latter may be prepared as described by Lewin, Wills, and Baron (1969) from the epoxy resin Araldite, commercially available in the Araldite adhesive pack, which contains both resin and hardener.

Results and Discussion

TEMPERATURE OF INCUBATION

Boyer, Baron, and Talalay (1965), using androsterone as substrate, showed that the reaction

rate of 3α-hydroxysteroid dehydrogenase rises essentially linearly with temperature (16-35°C). However, Turnberg and Anthony-Mote (1969) using sodium cholate as substrate found a fall-off in extinction at 340 nm at 37°C. Using sodium glycocholate as substrate and hydrazine hydrate as a ketone trapping agent, the reaction was found to be complete at 37°C in 45 min (Fig. 2). Similar results were obtained with equimolar solutions of other bile acids (Fig. 3) and no reversal of the NAD/NADH reaction was observed with any of the bile acids tested (Table I).

Standard Solution	*Concentration (μmol/litre)*	
	Calculated	*Estimated*
Sodium taurocholate	151·6	149·7
Sodium taurodeoxycholate	179·2	179·4
Sodium taurochenodeoxycholate	112·1	112·8
Sodium glycochenodeoxycholate	219·5	215·3
Sodium glycodeoxycholate	222·6	221·5
Sodium taurolithocholate	164·2	166·2
Sodium glycolithocholate	155·2	153·6
Cholic acid	222·7	235·8
Deoxycholic acid	187·0	185·9
Chenodeoxycholic acid	209·0	208·1
Sodium lithocholate	160·6	155·9

Table I *Comparison of analytical yield from aqueous solutions of different bile salts and bile acids*

Total Bile Acid in Serum (μmol/litre)	*Standard Used*	*Standard Added (μg)*	*Standard Recovered (μg)*	*Percentage Recovery*
1·4	Taurocholate	104·0	87·9	85
112·8	Taurocholate	104·0	91·5	88
174·0	Taurocholate	104·0	88·2	85
41·2	Glycocholate	73·5	61·0	83
236·0	Glycocholate	73·5	63·5	86
46·0	Lithocholate	60·0	51·3	86

Table II *Recovery experiment*

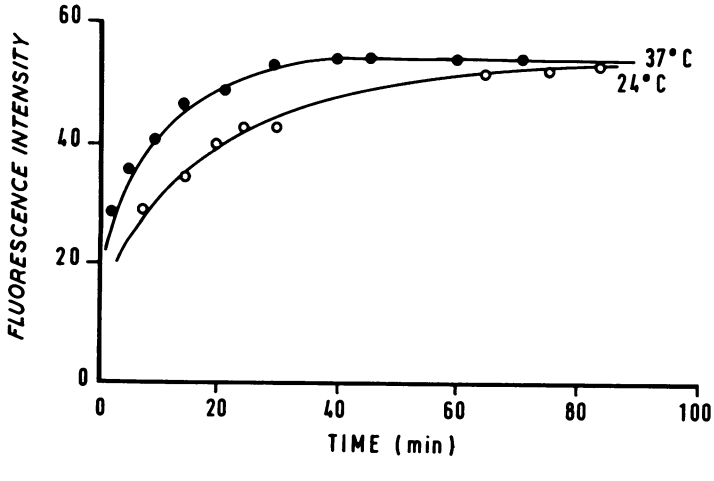

Fig. 2 *Development of fluorescence with time at 24°C and 37°C using 50 μl sodium glycocholate (200 μmol/litre) in the reaction mixture.*

RECOVERY

Table II shows the results of recovery experiments performed by adding a standard aqueous solution to serum before the extraction procedure. The recovery, 83-88%, was found to be independent of the original serum concentration and also of the bile acid used.

PRECISION

The precision of the method was determined as follows: (*a*) by considering the results of six replicate determinations. The mean value was 149·4 μmol/litre, the standard deviation was 4·4, and the coefficient of variation was 3%. (*b*) By considering the results of duplicates of duplicate determinations, performed on 10 different sera, from different batches, and ranging in bile acid concentration from 2·3 to 256 μmol/litre. The mean value of the results was 73·6 μmol/litre, the standard deviation was 3·1, and the coefficient of variation was 4·2%.

PRACTICAL RANGE OF MEASUREMENT

The smallest amount of NADH fluorimetrically detectable in a volume of 2 ml was 0·0005 μmol which gave a galvanometer deflection of approximately 2 units (galvanometer controls set at × 1,

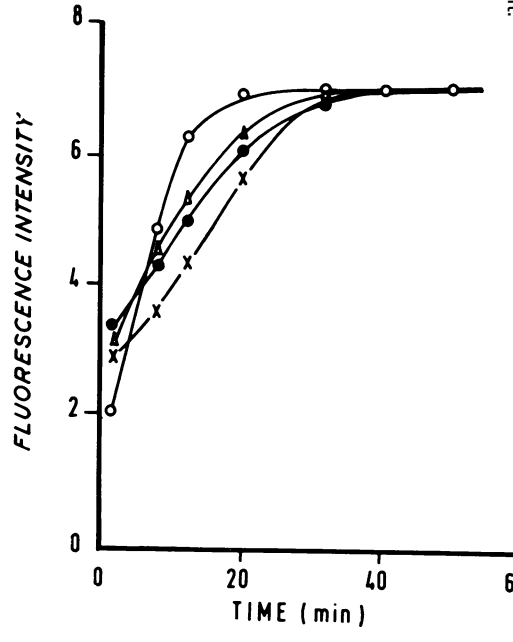

Fig. 3 *Development of fluorescence in reaction mixtures containing 50 μl bile salt solutions (24 μmol/litre) at 37°C in relation to time*

○——○ Sodium taurocholate
△——△ Sodium chenodeoxycholate
●——● Sodium glycocholate
×——× Sodium lithocholate

full scale deflection 14 units). This corresponds to a bile acid concentration in the alcoholic extract of 10 μmol/litre. Hence for low serum concentrations it is necessary to concentrate the serum during the extraction procedure 1 in 3 or 1 in 6. If the serum tests give higher readings than the top standard of 205 μmol/litre then the extracts should be diluted with 50% ethanol so as to avoid the quenching effects shown in Figure 1. Alternatively, as in the case of sera from patients with obstructive jaundice, smaller volumes of serum may be used, thus minimizing interference from bilirubin.

SPECIFICITY

By using an enzyme procedure with suitable blanks such problems as the necessity of separating bile pigments and phospholipids from the bile acids are minimized. However, the Sigma preparation is a mixture of 3α-hydroxysteroid dehydrogenase and 3,17β-hydroxysteroid dehydrogenases, so to check the specificity of the procedure, the results obtained using this preparation were compared with those obtained using pure 3α hydroxysteroid dehydrogenase. No significant difference between the two sets of results was observed thus demonstrating that in the above procedure only acidic steroids which were 3α hydroxysteroids were measured.

Method	*Material*	*Bile Acids (μmol/litre)*	*Recovery (%)*	*Author*
Gas liquid chromatography	Serum	0·6-4·6 (range)	59-87	Sandberg *et al* (1965)
Gas liquid chromatography	Serum	0·24-2·9 (range)		Makino, Nakagawa, and Mashimo (1969)
H_2SO_4/UV	Serum	20-44 (range)		Carey (1956)
Thin-layer chromatography + H_2SO_4/fluorimetry	Serum	<5 (range)	85-90	Panveliwalla *et al* (1970)
Enzyme spectrophotometry	Blood	4·4 ± 1·1 (mean ± 1 SD)	82	Iwata and Yamasaki (1964)
Enzyme fluorimetry	Serum	Male 0-4·7 (range) Female 1·0-8·2 (range)	83-88	Present method

Table III *Normal values for serum bile acids*

NORMAL VALUES

Blood was collected from 24 fasting subjects (12 female and 12 male), mainly medical students and radiography students. The serum total bile acids were estimated using the method described; the range for the male group was 0-4·7 μmol/litre, that for the female group 1·0-8·2 μmol/litre (Fig. 4). The normal range of total bile acids in serum found is similar to normal ranges taken from the literature (Table III).

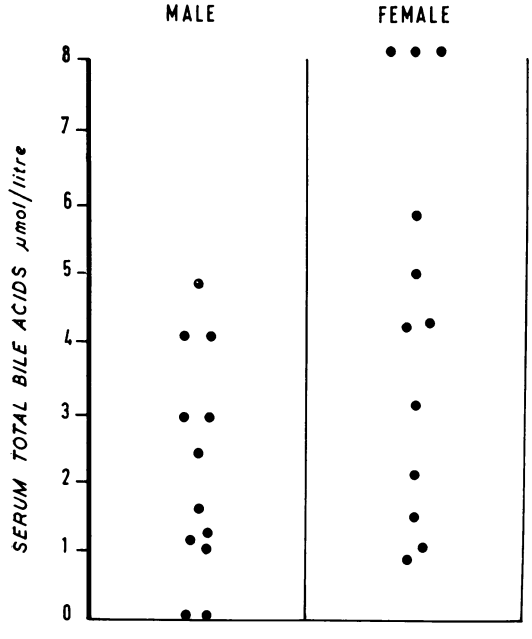

Fig. 4 *Normal values for 12 females and 12 males.*

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References

Boyer, J., Baron, D. N., and Talalay, P. (1965). Purification and properties of a 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*. *Biochemistry*, **4**, 1825-1833.

Carey, J. B., Jr. (1956). The serum dihydroxy-trihydroxy bile acid ratio in liver and biliary tract disease. (Abstr.) *J. clin. Invest.*, **35**, 695-696.

Frosch, B., and Wagener, H. (1967). Quantitative determination of conjugated bile acids in serum in acute hepatitis. *Nature (Lond.)*, **213**, 404-405.

Green, W. G. E., and Israelstam, G. F. (1968). Increased sensitivity in fluorimetric determinations of nicotinamide nucleotide coenzymes. *Canad. J. Biochem.*, **46**, 390-392.

Gregg, J. A. (1966). New solvent systems for thin-layer chromatography of bile acids. *J. Lipid Res.*, **7**, 579-581.

Hofmann, A. F. (1964). Thin-layer chromatography of bile acids and their derivatives. In *New Biochemical Separations* edited by A. T. James and L. J. Morris, pp. 261-282.

Hurlock, B., and Talalay, P. (1957). Principles of the enzymatic measurement of steroids. *J. biol. Chem.*, **227**, 37-52.

Iwata, T., and Yamasaki, K. (1964). Enzymatic determination and thin-layer chromatography of bile acids in blood. *J. Biochem. (Tokyo)*, **56**, 424-431.

Lewin, M. R., Wills, M. R., and Baron, D. N. (1969). Ultramicrofluorimetric determination of calcium in plasma. *J. clin. Path.*, **22**, 222-225.

Lowry, O. H., Roberts, N. R., and Kapphahn, J. I. (1957). The fluorimetric measurement of pyridine nucleotides. *J. biol. Chem.*, **224**, 1047-1064.

Makino, I., Nakagawa, S., and Mashimo, K. (1969). Conjugated and unconjugated serum bile acid levels in patients with hepatobiliary diseases. *Gastroenterology*, **56**, 1033-1039.

Marcus, P. I. and Talalay P. (1956). Induction and Purification of α & β Hydroxysteroid Dehydrogenases. *J. biol. Chem.*, **218**, 661-674.

Panveliwalla, D., Tabaqchali, S., Lewis, B., and Wootton, I. D. P. (1970). Determination of individual bile acids in biological fluids by thin-layer chromatography and fluorimetry. *J. clin. Path.*, **23**, 309-314.

Sandberg, D. H., Sjövall, J., Sjövall, K., and Turner, D. A. (1965). Measurement of human serum bile acids by gas-liquid chromatography. *J. Lipid Res.*, **6** 182-192.

Turnberg, L. A., and Anthony-Mote, A. (1969). The quantitative determination of bile salts in bile using thin-layer chromatography and 3α hydroxysteroid dehydrogenase. *Clin. chim. Acta*, **24**, 253-259.