Serum bile acid levels in hypercholesterolaemic patients

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SYNOPSIS A shortened method of estimating serum bile acids is described, based on extraction and the measurement of fluorescence in sulphuric acid solution at 37°C. The cholate and dihydroxy bile acid fractions are determined separately by measurements made one hour and 24 hours after preparing the solution.

About 1% of the serum cholesterol escapes separation and appears in the sulphuric acid solution; in its fluorescent properties it behaves in a manner almost identical with dihydroxy bile acids. As a result, the normal serum bile acids are overestimated by about 2 mg./100 ml. This effect is unimportant in cases of jaundice. In non-jaundiced cases with hypercholesterolaemia, the apparently raised serum bile acid levels are accounted for by the interference from cholesterol and there is no evidence that a raised serum cholesterol level is usually associated with raised serum bile acids.

The concentrations of cholate (3, 7, 12-trihydroxy-cholanate) and of cholesterol were measured by Friedman, Byers, and Rosenman (1952) in the serum of a number of patients. They found that rises in the serum cholesterol level were invariably accompanied by increases in the serum cholate level up to several times the normal. These patients did not have liver disease. Similar rises in serum chenodeoxycholate levels (3, 7-dihydroxycholanate) were reported by Wysocki, Portman, and Mann (1955). If confirmed, these findings are of great theoretical interest in view of the known production of bile acids from cholesterol (Bergström and Norman, 1953; Bergström and Lindstedt, 1956). However, methods for the estimation of bile acids in blood are often non-specific and cholesterol is an interfering substance in many of the reactions used.

Wootton and Osborn (1960) outlined a method of estimating di- and trihydroxy bile acids in blood which was designed to be as specific as possible. The blood extract was subjected to six stages of purification and separation before measurement and the results obtained by this method in cases of liver disease were reported by Osborn, Wootton, Da Silva, and Sherlock (1959).

For routine purposes, the method of Wootton and Osborn is impossibly long and complicated. A shorter but less specific method is described here together with a comparison of the results obtained by using the two methods. Using the shortened method, we have re-examined the serum bile acid

levels in hypercholesterolaemic patients, both jaundiced and not jaundiced.

METHODS

PREPARATION OF EXTRACT Serum (0.5 ml.) was added to a mixture of 25 ml. of ethanol and 1 ml. of Josephson's (1935) reagent (saturated barium hydroxide solution containing 0.4% barium acetate). The mixture was heated in a boiling water-bath for several minutes, allowed to stand overnight, and centrifuged. The supernatant fluid was evaporated to dryness under vacuum in a rotary evaporator. The residue was dissolved in 18 ml. of 97.5% (v/v) acetic acid, transferred to a 100 ml. separating funnel, and subjected to a three-stage counter-current distribution using phases of 18 ml. of 97.5% acetic acid and 15 ml. of redistilled *n*-heptane (Ahrens and Craig, 1952).

The combined acetic acid phases were evaporated to dryness under reduced pressure, the residue was dissolved in 5 ml. 2*N*-potassium hydroxide, and bile acid conjugates were hydrolysed by heating the solution under pressure at 105 to 110°C. for 18 hours. The cooled solution was acidified to pH 2 by the addition of 4*N*-hydrochloric acid and extracted three times with ether. The combined ether extracts were evaporated to dryness and the flask heated to 80 to 90°C. for three hours. After removal from the oven, and while still warm to the touch, 25 ml. concentrated sulphuric acid previously warmed to 37°C. was added and swirled round the flask to dissolve the residue quickly. The sulphuric acid solution was used for measurement of fluorescence.

FLUORESCENCE MEASUREMENTS A filter fluorimeter was used (Laurence, 1957). The incident light from a mercury arc-lamp was filtered through Chance OB 10 glass. The

fluorescent light was measured with a photomultiplier screened with a Chance OY 6 yellow-green filter. Spectrophotofluorimetric measurement showed that the excitation and fluorescent spectra had maxima for cholate at 395 and 425 $m\mu$ respectively and for chenodeoxycholate or deoxycholate at 405 and 435 $m\mu$.

The intensity of the fluorescence of a sulphuric acid solution of bile acid depends on temperature and time. In general, fluorescence increases with time and develops more quickly in a warmer solution. It was therefore necessary to maintain the solution at a constant temperature, and in this work 37°C. was chosen as the standard temperature. When repeated measurements are made on solutions kept at this temperature, the fluorescence of cholic acid shows a relatively small increase with time whereas that of the dihydroxy acids is about two to three times as intense at 24 hours as at one hour (Fig. 1). This offered a method for estimating the components of a mixture of dihydroxy and trihydroxy acids. Measurements were made after one hour and 24 hours' incubation of the sulphuric acid solution at 37°C.

If x is the fluorescent light output at one hour from the cholate in the solution and y is that from the dihydroxy acids at the same time, then

$$\begin{aligned}x + y &= R_1 \dots\dots\dots 1 \\1.1x + 2.3y &= R_{24} \dots\dots\dots 2\end{aligned}$$

where R_1 and R_{24} are the intensities of fluorescence at one and 24 hours respectively, measured in arbitrary units. The factors 1.1 and 2.3 are derived from measurements of pure standard solutions, the latter coefficient being correct only for pure chenodeoxycholate. However, even if deoxycholate constituted the entire dihydroxy bile acid fraction of the solution, the error involved in using the same coefficient would only be about 10%. In any analysis the contributions from cholate and dihydroxy bile acids to the measured fluorescence were calculated from equa-

tions 1 and 2; the absolute amounts of each could then be derived by reference to the fluorescence of known standard solutions.

The accuracy of this differential analysis was confirmed by measuring the fluorescence of known mixtures of pure bile acids. In a series of 15 such experiments, with an amount of bile acid present equivalent to a serum level of 10 mg./100 ml., the worst result was 6.5% in error.

STANDARDS AND BLANKS Recovery experiments carried out by adding known amounts of bile acids to serum samples proved that about 60% to 70% of the added bile acid was present in the sulphuric acid solution. In different batches, the proportion lost was highly constant. To correct for incomplete recovery, a standard was prepared by dissolving 20 mg./100 ml. of sodium glycocholate in a pooled normal serum and carrying the standard and a blank of the same pooled serum through the complete procedure with each batch.

EVALUATION OF METHOD Because of uncertainty about the specificity of this shortened method, and in particular because the fluorescent properties of cholesterol are almost identical quantitatively and qualitatively with those of chenodeoxycholate (Fig. 1), experiments with cholesterol added to extraction mixtures containing serum were carried out. Table I indicates the result of such a recovery experiment. It is evident that about 1 to 1½% of the initial concentration of cholesterol came through to the final stage. There was probably some further interference from phospholipids.

The overall validity of the method was therefore further checked by comparing the results obtained in various clinical conditions with those already reported by Osborn *et al.* (1959) who used the long method of higher specificity. Analysis of variance (Table II) indicated that the present method generally overestimated the bile acid content by 1 to 4 mg./100 ml. It can therefore be considered satisfactory for use only in cases where there is a raised serum bile acid level.

FIG. 1. The increase in fluorescence of sulphuric acid solutions with time. The compounds were present at a concentration of 4 $\mu\text{g./ml.}$

1, deoxycholate; 2, cholesterol; 3, chenodeoxycholate; 4, cholate.

TABLE I

EFFECT OF ADDED CHOLESTEROL ON THE DIHYDROXY BILE ACID RESULT

Cholesterol Added to 0.5 ml. Serum (mg.)	Apparent Increase in Dihydroxy Bile Acids (mg./100 ml.)	Proportion of Added Cholesterol Remaining in Final Solution (%)
2	5.6	1.4
4	13.6	1.8
5	6.4	0.7
5	9.2	1.0

RESULTS

Twelve specimens of normal serum were examined. The mean cholesterol level was 193 mg./100 ml. The mean serum cholate value was 2.0 mg./100 ml. (standard deviation 1.2 mg./100 ml.) and the mean dihydroxy bile acid concentration was 3.4 mg./100 ml. (standard deviation 1.4 mg./100 ml.) On the basis of the results quoted in Table I, approximately 2 mg./

TABLE II

COMPARISON OF RESULTS BY LONG METHOD OF WOOTTON AND OSBORN (1960) AND PRESENT SHORT METHOD					
Clinical Condition	Method	No. of Cases	Mean Bile Acid Level (mg./100 ml.)	Variance Ratio (F)	Fiducial Probability (P)
Acute obstructive jaundice	Long	6	15.4	8.4	> 0.2
	Short	16	14.1		
Chronic obstructive jaundice	Long	11	15.0	5.9	> 0.2
	Short	16	17.1		
Virus hepatitis	Long	9	8.7	2.2	0.2 > P > 0.1
	Short	7	12.9		
Cirrhosis	Long	24	6.0	1.2	> 0.2
	Short	16	7.8		

TABLE III

ANALYTICAL RESULTS ON HYPERCHOLESTEROLAEMIC SERA

Diagnosis	Cholesterol (mg./100 ml.)	Cholate (mg./100 ml.)	Dihydroxy Bile Acids (mg./100 ml.)
Myxoedema	470	5.0	7.9
Polyarteritis nodosa	530	4.8	7.8
Idiopathic hypercholesterolaemia	420	3.3	5.9
Myxoedema	470	3.5	6.4
Sarcoidosis	380	3.5	5.0
Myxoedema	440	2.3	4.2
Idiopathic hypercholesterolaemia	360	3.7	6.6
Myxoedema	440	1.3	3.0
Myocardial ischaemia	830	4.5	7.4
Myocardial ischaemia	330	1.0	2.2
Idiopathic hypercholesterolaemia	300	4.3	6.7
Acute porphyria	440	1.8	3.4
Myxoedema	535	4.6	7.9
Nephrosis	385	3.7	5.4
Secondary carcinomatosis	300	3.3	5.9
Idiopathic hypercholesterolaemia	300	3.4	4.2
Cushing's syndrome	365	3.6	6.6
Kimmelstein-Wilson syndrome	400	3.2	5.7
Idiopathic hypercholesterolaemia	340	2.7	4.3

100 ml. of the apparent dihydroxy bile acid can be attributed to the retained cholesterol. The total serum bile acid of a normal subject is thus 3 to 4 mg./100 ml. This finding agrees with those in several series (Sherlock and Walshe, 1948; Friedman, Byers, and Rosenman, 1952; Sobel, Goldberg, and Slater, 1953; Carey, 1956), although it must be admitted that other authors have reported very much higher values for the normal serum bile acid (for review, see Wootton and Osborn, 1960).

When the method described here was applied to 19 serum specimens with raised cholesterol concentrations rather higher values for bile acids were obtained (Table III). The specimens were not jaundiced and had cholesterol concentrations in the range 300 to 850 mg./100 ml. The increases in bile acids were mainly in the dihydroxy bile acid component and were clearly related to the serum cholesterol level shown in Figure 2. The line in this figure is drawn through the centre of mass of the points with the slope which would result from a retention of 1% of the serum cholesterol. It is evident that the values found can be explained by the imperfect separation of cholesterol in the analytical procedure.

DISCUSSION

The method of bile-acid estimation described above is admittedly imperfect; complete removal of cholesterol is not achieved, and as a result, the dihydroxy bile acids in particular are overestimated. On the other hand, the method is reasonably convenient in use and the errors are unimportant in conditions associated with raised serum levels, as shown by the analysis of variance in Table II.

When samples of serum containing increased amounts of cholesterol were examined, there was a general tendency for the apparent dihydroxy bile acid content to be increased above normal. In general the higher the cholesterol level the higher was the result for bile acid, a situation very similar to that reported by Friedman *et al.* (1952). However, the regression line in Fig. 2 indicates that the apparent connexion between serum levels of these two constituents can adequately be explained by the residual small proportion of cholesterol which remained in the extracts used for fluorimetry.

This raises the question of whether the results of Friedman *et al.* (1952) and Wysocki *et al.* (1955)

FIG. 2. Relation between serum dihydroxy bile acid concentration and serum cholesterol level in 19 cases with raised serum cholesterol levels.

could have a similar explanation. The latter used the method described by Minibeck (1938) which involved the development of fluorescence in sulphuric acid solution in a similar way to the present method and might therefore be expected to be sensitive to cholesterol contamination. Friedman and his associates removed cholesterol and other lipids by treating a serum extract with calcium and barium hydroxides, followed by solution in sulphuric acid and a measurement of absorption at $385\text{ m}\mu$ as described by Wilken (1937). Although they stated

that their values were not influenced by a raised serum cholesterol level, they do not provide quantitative evidence, and the present work indicates that the bile acid level may be seriously overestimated if removal of cholesterol is not perfect. It seems most likely that previous reports of raised bile acid levels in hypercholesterolaemic patients are in error, and the results reported can be explained as due to a lack of specificity of the methods used.

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