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Colorimetric enzymatic measurement of serum total 3α-hydroxy bile acid concentrations without extraction

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SUMMARY A new method for the simpler colorimetric assay of serum total 3α-hydroxy bile acid concentrations without prior extraction has been investigated. The lowest concentration that could be reliably measured was 3 μmol/l and the results obtained were in agreement with those obtained using extraction and the spectrofluorimetric enzymatic assay ($y = 1{\cdot}007x - 1{\cdot}86$, $r = 0{\cdot}99$, $n = 36$). Results for 20 normal sera collected after an overnight fast were 6·2 ± 5·1 μmol/l (mean ± SD) and in 20 normal plasma randomly collected were 4·9 ± 4·2 μmol/l. This new assay is comparable in specificity and sensitivity to the older spectrofluorimetric enzymatic assays and convenient for routine use.

The measurement of serum bile acid concentrations has long been regarded by many authors as providing a very sensitive index of hepatobiliary function.[1–4] However, this test has yet to be accepted by clinical chemists and added to their routine assays for monitoring liver function. There are two reasons for this failure—lack of confidence in the specificity and sensitivity of serum bile acid measurements with regard to the detection of early liver disease[5 6] and, of equal importance, lack of a method which fulfils the usual analytical reliability criteria and which could be readily applied in the clinical chemistry laboratory. This present report deals solely with the methodological problems and considers a possible solution in terms of an assay kit which has recently become commercially available.

The kit makes use of the following reactions:[7]

$$\text{3}\alpha\text{-hydroxy bile acid} + \text{NAD} \xrightarrow[\text{dehydrogenase}]{3\alpha\text{-hydroxysteroid}} \text{3-keto bile acid} + \text{NADH}$$

$$\text{NADH} + \text{chromogen (NBT)} \xrightarrow{\text{Diaphorase}} \text{Chromophore (Formazan)} + \text{NAD}$$

The use of hydroxysteroid dehydrogenase for the determination of serum bile acid concentration is not new, normal values using spectrophotometry being first reported in 1964[8] and those obtained with the more sensitive spectrofluorimetric assay in 1970.[9] However, the linkage of the product NADH to a chromogen (nitrobluetetrazolium) to generate a chromophore provides a spectrophotometric end point with a sensitivity approaching that of the spectrophotometric assay but without the necessity of serum extraction. This direct and immediate assay thus represents a major advance in serum bile acid methodology.

Our primary concern therefore was to investigate the claims for this kit of sensitivity and specificity. To do this we largely followed a recommended scheme for the evaluation of kits in the clinical chemistry laboratory.[10]

Material and methods

NYCO-HSD-COL KIT METHOD

The kit was supplied by Nyegaard and Co, Oslo, Norway, and consisted of:

(1) Sample reagent—which contains all the components necessary for the generation of the chromaphore by bile acids and includes a serum inactivator,

(2) Blank reagent—which contains all the constituents of the sample reagent with the exception of the hydroxysteroid dehydrogenase,

(3) Sodium phosphate buffer, pH 7·0, 65 mmol/l (once reconstitued in this buffer, sample and blank reagents are stable for at least two weeks at 4°C),

(4) Bile acid standard mixtures prepared in bile acid free bovine serum, of glycolate, glycodeoxy-

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cholate and taurochenodeoxycholate, to final total bile acid concentrations of 10, 50 and 100 μmol/l, (5) Stop reagent—100 mmol/l HCl containing surfactant.

To perform the assay 500 μl of sample reagent reconstituted in the phosphate buffer was added to 200 μl each plasma and standard for the tests and for the blanks 500 μl blank reagent was used. After 10 min incubation at 37°C, 500 μl stop reagent was added to both test and blank tubes. The absorbance of each tube was read at 540 nm, a standard curve drawn, and results calculated in the usual way.

SPECTROFLUORIMETRIC ENZYMATIC ASSAY

This was performed as previously described[9] with the modification that methanolic extracts of serum bile acids were prepared using Amberlite XAD-2 in the batch procedure.[13]

Results and discussion

SENSITIVITY

To examine the sensitivity of the proposed method analyses of doubling dilutions of the kit 50 μmol/l standard were performed in duplicate on three occasions (Fig. 1). The range of absorbance obtained for each solution was small (coefficient of variation <2%) but it was not possible to distinguish between concentrations of ≤3·1 μmol/l. The limit of detection of this method was therefore regarded as 3 μmol/l.

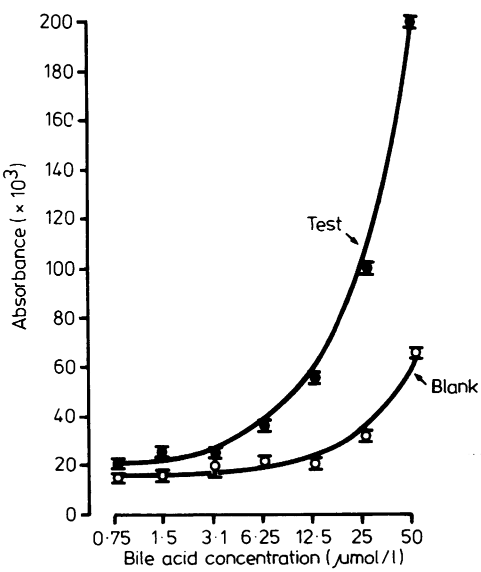

Fig. 1 *Sensitivity. Absorbance (mean ± SD) generated by dilutions of 50 μmol/l standard solution.*

Whereas this sensitivity is comparable with that of the original spectrofluorimetric enzymatic technique[9] it is much less than any of the subsequent modifications.[11–19] It is unlikely, however, that the sensitivity of the new method will be shown to be inadequate in any clinical application. In particular, reductions in serum bile acid concentrations associated with gastrointestinal disease are more evident post-prandially than in the fasting state,[20 21] and following meals total serum bile acid concentrations are normally above 3 μmol/l.[3 4 12]

PRECISION

The "within-run" precision[10] was estimated from the results of duplicate determinations on 37 different sera. The duplicate samples were randomly distributed throughout the run. For concentrations ≤10 μmol/l, the coefficient of variation was 16% (7·2 ± 1·1 (mean ± SD, n = 20)), for those 10–25 μmol/l the CV was 10% (14·6 ± 1·5; n = 9), and for those >25 μmol/l the CV was 6·8% (63·1 ± 4·3; n = 8).

The "between-day" precision[10] was estimated from the results obtained on three sera each analysed in duplicate on 17 consecutive days. The CV at <10 μmol/l was 12% (5·8 ± 0·7), between 11–25 the CV was 10% (31·7 ± 3·2), and at >25 μmol/l the CV was 2% (105 ± 2·1).

Analysis of the "control" serum supplied with the kit gave results with CV of 9% (39·9 ± 3·6).

SERUM INTERFERENCE

In order to evaluate possible interference in this assay from serum constituents other than bile acids, the following experiments were performed. Two sera were diluted with isotonic saline, and the dilutions analysed using the kit. The results obtained on each of the dilutions were expressed as a percentage of the concentration found in the corresponding undiluted serum. Whilst the results for undiluted, 1/2 and 1/4 dilutions were linear and parallel to the standard curve, recoveries from the 1/8 dilution were poor (Table). To investigate this further, standard solutions of taurocholic acid were prepared in water and in serum. Although both sets of results were linear, parallelism with the standard curve was only found in the presence of serum (Fig. 2). It was concluded that there is in serum a factor which enhances the reaction and that if the method is to be applied to unextracted sera, the dilutions of sera must be less than 1/4. (This caution may be particularly relevant to analyses of bile and bile-rich duodenal contents with this kit and aqueous solutions of standards of appropriate concentrations should be used—Barbara H Billing, personal communication 1983.)

Effect of dilution in isotonic saline

Dilution	*Serum no*	
	1	*2*
Neat	57·6 μmol	160 μmol
1/2	103%	99%
1/4	102%	99%
1/8	55%	88%

COMPARISON WITH THE ENZYMATIC FLUORIMETRIC METHOD

The results obtained using the spectrophotometric assay kit were compared with those obtained on the same samples using the enzymatic fluorimetric assay.[9] For that procedure, bile acids were extracted from serum using Amberlite XAD-2 using the batch procedure and the fluorimetric assay applied to the methanolic extracts with little modification from the method originally described.[9] Samples which were haemolysed or which had become turbid on storage at −20°C were excluded from this study. The results obtained using the kit were usually lower than those obtained on the same sample using the spectrofluorimetric assay and the two sets of data were far from identical. The correlation, however, between the results of the two methods was satisfactory (Fig. 3).

NORMAL VALUES

Results in sera obtained from 20 laboratory staff who had fasted overnight were 6·2 ± 5·1 μmol/l (mean ± SD; range 3–16·3 μmol/l). Results in plasma (sodium citrate), randomly collected in relation to meal times, from staff who were acting as controls in another investigation were 4·9 ± 4·2 (mean ± SD; range 3–15·1 μmol/l). There is some disagreement in the literature concerning the normal range of serum bile acid concentrations using enzymatic fluorimetric techniques. Some groups report normal ranges similar to those obtained by the present spectrophotometric technique in apparently fasting subjects;[13 22 23] others find these higher values only in non-fasting subjects.[4 14 24] Perhaps the real difficulty lies in assuming that no contraction of the gallbladder or movement of the bile acid pool occurs during fasting.

Of all the methods (enzymatic, radioimmunoassay, gas chromatography, gas chromatography—mass spectrometry, high pressure liquid chromatography—mass spectrometry) currently used to measure serum bile acid concentration, the enzymatic method is the least time consuming, complicated and expensive. Our conclusion is that this colorimetric enzymatic method for serum total

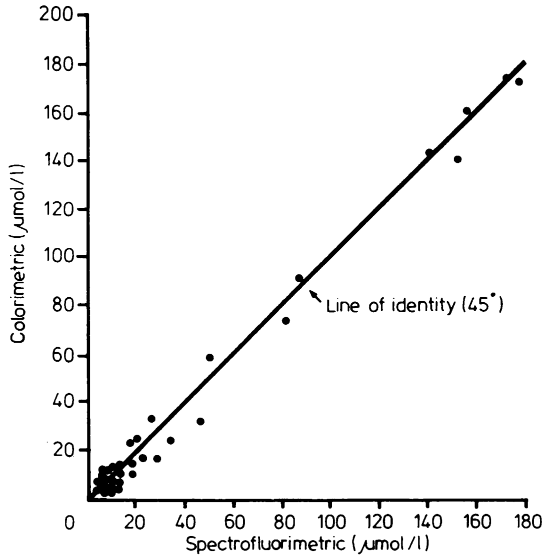

Fig. 3 *Serum total bile acid concentrations (μmol/l). Colorimetric v spectrofluorimetric assay (y = 1·007x − 1·86, r = 0·993).*

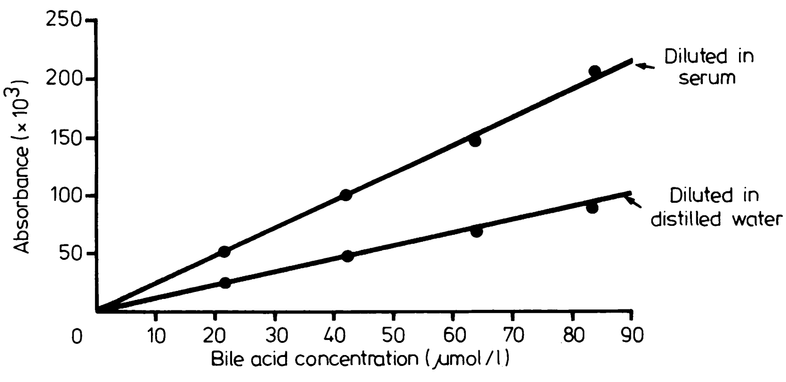

Fig. 2 *Standard solutions. Absorbance generated by standard solutions of taurocholate prepared in either water or serum as described in text.*

bile acid measurement is comparable to the older fluorimetric enzymatic assays in specificity and more suitable for routine use.

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