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# Microassay of Serum Bile Acids by an Enzymatic Cycling Method[1)]

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A new colorimetric method for the determination of total 3α-OH bile acids in serum by means of an enzymatic cycling reaction is described. Bile acids were oxidized by 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*, and after the destruction of the remaining nicotinamide-adenine dinucleotide $(NAD)^+$, the formed NADH was determined. The cycling system consisted of two enzymes, alcohol dehydrogenase from yeast and diaphorase from *Clostridium kluyverii*. The formed NADH was oxidized by diaphorase in the presence of 3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride and then was reduced again by alcohol dehydrogenase and ethanol. Subsequently, formazan produced as a final product was determined by rate assay. As the proposed method does not require as extraction step, it is simpler and more accurate than the conventional methods.

**Keywords**——bile acid; serum; cycling reaction; diaphorase; 3α-hydroxysteroid dehydrogenase; alcohol dehydrogenase

## Introduction

In recent years, the determination of serum bile acids has been considered a useful aid in the diagnosis of hepatobiliary diseases.[2,3)] Enzymatic methods based on the spectrophotometric measurement of NADH formed by the oxidation of bile acids with 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* have been developed.[4–6)] These enzymatic methods require large amounts of serum, because the content of bile acids in serum is very small. For clinical tests, accuracy and simplicity are required, and the step of extraction of bile acids from serum, which is complex and time-consuming, results in a low recovery.[7)]

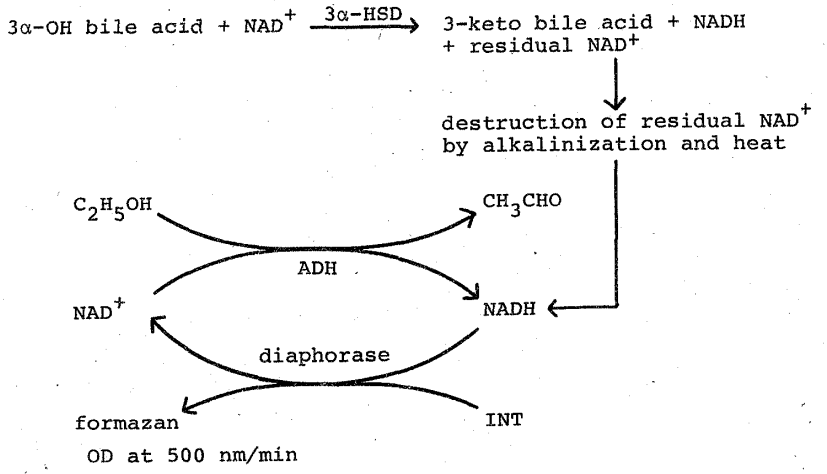


Fig. 1. Principle of the Enzymatic Microassay of 3α-OH Bile Acids using a Cycling System for the Amplification of NADH

3α-HSD, 3α-hydroxysteroid dehydrogenase; INT, 3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride; ADH, alcohol dehydrogenase.

On the other hand, an enzymatic cycling system require only a small volume and is very sensitive. So far, a spectrophotometric assay of serum bile acids based on an enzymatic cycling reaction has been developed.[8] However, there is still room for improvement in the extraction step of this cycling method.

Therefore, we developed a direct enzymatic cycling method using alcohol dehydrogenase and diaphorase as shown in Fig. 1.

## Materials and Methods

**Reagents and Enzymes**——3α-Hydroxysteroid dehydrogenase (3α-HSD; *Pseudomonas testosteroni*) was obtained from Daiichi Pure Chemicals Co., Ltd., alcohol dehydrogenase (Yeast) from Boehringer Mannheim Co. and diaphorase (*Clostridium kluyverii*) from Worthington Biochemical Co. 3-(*p*-Iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT), bovine serum albumin and ethanol were purchased from Wako Pure Chemical Industries, Ltd., hydrazine hydrate from Tokyo Chemical Industry Co., Amberlite XAD-2 from Rohm and Haas Co., glycodeoxycholic acid sodium salt and taurodeoxycholic acid sodium salt from Sigma Co. Cholic acid, chenodeoxycholic acid and taurodeoxycholic acid were kindly supplied by Tokyo Tanabe Co., Ltd. Other reagents were of analytical grade.

**INT Buffer**——Tris-HCl buffer (0.1 M, pH 8.0) containing 2.0 mM INT, 0.02% (w/v) bovine serum albumin and 300 mM ethanol was used.

**Tris-Hydrazine Buffer**——Tris-HCl buffer (0.2M, pH 9.5) containing 2.7 mM EDTA-2Na, 300 mM hydrazine hydrate and 7.5 mM HCl was used.

**3α-HSD Solution**——3α-HSD (0.012 I.U.) was dissolved in 1 ml of Tris-hydrazine buffer containing 1 mM $NAD^+$.

**Standard Solution**——Glycocholic acid (20 μl) was dissolved in 7% (w/v) bovine serum albumin solution.

**Cycling Enzyme Solution**——Alcohol dehydrogenase (125 I.U.) and diaphorase (90 I.U.) were dissolved in 1 ml of distilled water.

**Standard Procedure for the Determination of Serum Bile Acids**——Fifty μl of the serum was placed in each of two mini tubes with a cap (6 × 50 mm) and 50 μl of 0.1 M NaOH was added to each tube in order to inactive $NAD^+$-dependent enzyme in the serum. Fifty μl of 3α-HSD solution was added to one tube (serum test) and 50 μl of Tris-hydrazine buffer containing 1 mM $NAD^+$ was added to the other (serum blank) and they were incubated at 37°C for 10 min. After 50 μl of 0.1 M bicarbonate buffer (pH 10.1) had been added to each tube, excess $NAD^+$ was destroyed by heating at 60°C for 30 min. The cycling procedure was as follows: 50 μl of the above reaction mixture from each tube was added to 1.9 ml of INT buffer and preincubated at 25°C for 3 min. Then, 50 μl of cycling enzyme solution was added to each tube and the reaction rate was measured at 500 nm and 25°C with a Shimadzu CL-720 micro-flow spectrophotometer. For the calibration curve of total 3α-OH bile acid, glycocholic acid was used as a standard.

**Conventional Method**——The total bile acids in the serum were extracted by the Amberlite XAD-2 batch method of Henegouwen and Hofman[7] and measured according to the manual supplied with Sterognost-3α (total bile acids test kit).

## Results

### Conditions of Enzyme Cycling Reaction

It was found that 0.6 mU of 3α-HSD completely oxidized 100 μM glycocholic acid within 10 min at 37°C, and the conditions for destruction of $NAD^+$ were examined without bile acids. By the proposed method, more than 99.96% of excess $NAD^+$ was destroyed by the addition of 50 μl of 0.1 M bicarbonate buffer (pH 10.1) and by heating for 30 min at 60°C. The optimum temperature of this cycling reaction was found to be 25°C. The sensitivity in the enzyme cycling reaction was dependent on the cycling frequency, which could be expressed as a function of $V_{max}$ and $K_m$ of alcohol dehydrogenase and diaphorase. The cycling frequency of 25 cycle/min was suitable for the determination of serum bile acids.

Under the above conditions, a linear relationship between the bile acids concentration and $OD_{500nm}$/min was obtained in the range of 2 to 100 μM.

### Recovery and Precision

The recoveries of 20 μM and 50 μM glycocholic acid added to 14 different sera were 101±4.3% and 97±4.5%, respectively. The reproducibility of the proposed method was also tested

using sera with different concentrations of bile acids, 6.1 μM, 17.1 μM and 58.9 μM. The coefficient of variation (C.V.) for within-day assay precision was below 5%. The day-to-day precision was 4.7% (C.V.) for 7 days using serum containing 24 μM bile acids.

Furthermore, various substances such as glucose, uric acid, ascorbic acid, bilirubin and albumin did not interfere with the proposed method.

## Comparison of the Proposed Method with the Conventional Method

In order to compare the proposed method with the enzymatic fluorometric method, the bile acids were extracted and then determined by means of the enzymatic kit Sterognost-3α. As shown in Fig. 2, a good correlation ($r$= 0.990) was observed between the proposed method and the enzymatic method.

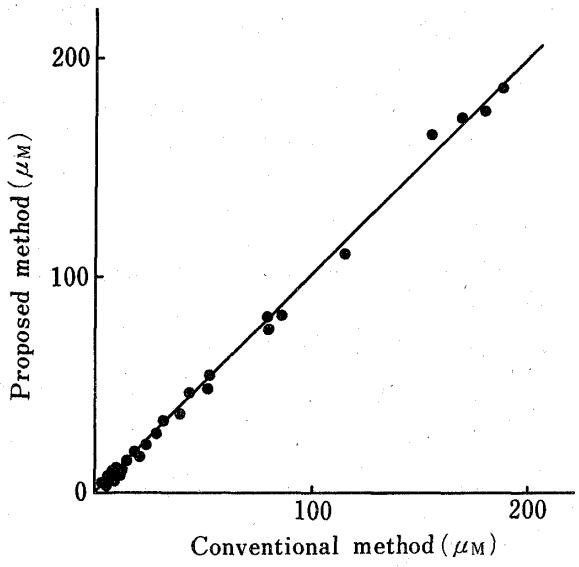


Fig. 2. Correlation of the Results of Serum 3α-OH Bile Acids Determination by the Proposed Method and the Conventional Method

$r = 0.990$
$Y = 1.08X + 2.38$
$n = 28$

## Discussion

The sensitivity of the proposed method is about 100 fold higher than that of the conventional method.[5] The sample volume used is very small and determination can be carried out by direct rate assay. In assay procedures, an extraction step is generally timeconsuming and results in low recovery as described by Henegouwen and Hofman[7] and Bradlow.[9] Recently, Nicolas *et al.*[8] reported an enzymatic cycling method for the determination of serum bile acids, in which deproteinization of serum with methanol–HCl was necessary before the enzyme cycling reaction. However, the present direct method has no extraction step and the time required for analysis is very short. The specificity of this method is dependent on the specificity of 3α-HSD. Five kinds of bile acids (see "Materials and Methods") were oxidized by 3α-HSD, but sulfate bile acids could not be oxidized.

By the addition of hydrazine to the buffer, the oxidation rate of bile acid by 3α-HSD was increased somewhat and the presence of a thousand-fold excess of NADPH in the cycling system had no effect on the determination because alcohol dehydrogenase from yeast is specific for NAD(H). Residual $NAD^+$ in NADH was completely destroyed under the proposed conditions and the blank rate with the serum sample was only 0.004 $OD_{500\ nm}$/min. Dehydrogenases existing in the serum was immediately inactivated by the addition of alkaline solution, and this step reduced the blank rate value. Bile acids in more than 30 healthy control sera were determined by the proposed method and the normal range (2 to 10 μM) was similar to that reported by Osuga *et al.*[10]

From these observation, the procedure of this cycling system, which permits direct measurement, seems to be simpler than that of the previously reported method,[8,11,12] and this method may also be used as a detection system in conjunction with the high pressure liquid chromatography technique.[13]

### References and Notes

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