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Microassay of Serum Androsterone by an Enzymatic Cycling Method[1)]

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A method for the analysis of androsterone sulfate in serum by means of an enzymatic cycling reaction was developed. Androsterone sulfate was solvolyzed, extracted and purified on Sephadex LH-20, then androsterone was oxidized by 3α-hydroxysteroid dehydrogenase from *Pseudomonas tetstosteroni* and the resulting NADH was determined by an enzymatic cycling method. The cycling system consisted of two enzyme, alcohol dehydrogenase from yeast and diaphorase from *Clostridium kluyverii*, and resazurin was used as an electron acceptor for diaphorase. Subsequently, resorfin formed from the resazurin was determined by the rate assay method at Ex_{560}, Em_{580} and 25°C. A linear relationship was obtained between the amount of androsterone and ΔF/min in the range from 0.035 to 3.5 μg/ml. The recovery, the reproducibility and the correlation between the proposed method and the conventional method using gas–liquid chromatography were also good. It appears that this simple and sensitive method could be useful as a clinical diagnostic test.

Keywords——determination of androsterone; alcohol dehydrogenase from yeast; diaphorase from *Clostridium kluyverii*; 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*; enzymatic cycling reaction

Androsterone is a metabolite of both testosterone and dehydroepiandrosterone and exists in serum largely as the sulfate ester. Androsterone is rapidly conjugated with sulfuric acid and glucuronic acid in the circulatory system, but almost all of the latter conjugate excreted by the renal mechanism. Therefore, androsterone present in the circulatory system is mostly the sulfate ester. Recently, it has been reported that the androsterone sulfate concentration in blood and urine changes in various diseases,[2)] especially in hypo- and hyperthyroidism,[3)] and the determination of androsterone sulfate has been regarded as being of importance in clinical diagnosis. Up to now, the measurement of serum androsterone sulfate has been performed by radioimmunoassay[4)] or gas-liquid chromatography.[5)] However, the radioimmunoassay for the determination of androsterone is not satisfactory in terms of safety and the necessity for the preparation of specific antibody. In the case of gas–liquid chromatography,

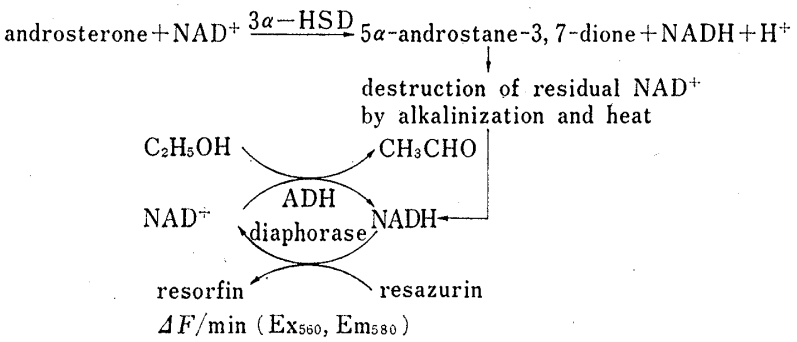

Fig. 1. Principle of the Enzymatic Microassay of Androsterone using a Cycling System for the Amplification of NADH

3α-HSD: 3α-hydroxysteroid dehydrogenase; ADH: alcohol dehydrogenase.

a large amount of the serum is required and the procedure is very complicated. Because the androsterone concentration in serum is very low, the method for the determination of bile acids using 3α-hydroxysteroid dehydrogenase (3α-HSD)[6] can not be directly applied to the determination of androsterone in serum. In this paper, we describe a simple and sensitive assay for the analysis of serum androsterone sulfate in a small volume of the serum. The principle of the proposed method is shown in Fig. 1. After the serum androsterone sulfate is solvolyzed, androsterone is extracted and oxidized by 3α-HSD in the presence of NAD^+.[7] Then the residual NAD^+ is selectively destroyed and the formed NADH is determined by means of an enzymatic cycling reaction.[8] NADH is oxidized to NAD^+ by alcohol dehydrogenase and ethanol and reduced again to NADH by diphorase and resazurin. The increase of fluorescence intensity of resorfin is measured.

Materials and Methods

Reagents and Enzymes——3α-Hydroxysteroid dehydrogenase (3α-HSD; *Pseudomonas testosteroni*) was obtained from Daiichi Chemical Co., alcohol dehydrogenase (yeast) from Boehringer Mannheim Co. and diaphorase (*Clostoridium kluyverii*) from Worthington Co.

Resazurin sodium salt, androsterone, pregnanediol (5β-pregnane-3α,20α-diol) and 5α-cholestane were purchased from Nakarai Chemical Co., and androsterone sulfate sodium salt, etiocholanolone (3α-hydroxy-5β-androstan-17-one), androstanediol (5α-androstane-3α,17β-diol), glycodeoxycholic acid sodium salt and taurodeoxycholic acid sodium salt were from Sigma Co. Cholic acid, deoxycholic acid, chenodeoxycholic acid, glycocholic acid, taurocholic acid, glycochenodeoxycholic acid and taurochenodeoxycholic acid were kindly supplied by Tokyo Tanabe Co. Bovine serum albumin (fraction V) was from Wako Pure Chemical Industries Ltd. (Japan), hydrazine hydrate from Tokyo Chemical Industry Ltd. (Japan), NAD^+ and NADH from Oriental Yeast Co., Sephadex LH-20 from Pharmacia Fine Chemicals Co., aluminum oxide and silica gel G from Merck Co. Other reagents were all of analytical grade and the organic solvents were distilled prior to use.

Cycling Buffer——Bovine serum albumin (20 mg) and 1.5 ml of ethanol were dissolved in 0.1 M Tris–HCl buffer (pH 8.0) to make 100 ml.

Tris-hydrazine Buffer——A solution of 363.4 mg of Tris, 150 μl of hydrazine hydrate and 11.2 mg of EDTA-2Na in distilled water was prepared and the pH was adjusted to 9.5 by the addition of 1 N HCl. The total volume was made up to 20 ml.

3α-HSD Solution——2.3 mU of 3α-HSD was dissolved in 1 ml of Tris–hydrazine buffer containing 0.5 mM NAD^+.

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

Standard Solution——Androsterone sulfate was dissolved in distilled water to 0.58 μg/ml.

Extraction and Solvolysis of Androsterone Sulfate——The extraction and solvolysis were performed according to the method of Kream *et al.*[4b] using 50—200 μl of serum.

Sephadex LH-20 Column Chromatography of Androsterone——The dried extract was dissolved in 0.2 ml of chloroform: *n*-heptane: ethanol (50: 50: 1, water to saturation)[9] and applied to a Sephadex LH-20 column (70 × 150 mm) which was packed with 1.5 g of gel. Elution was performed with the same solvent system at a flow of 30 ml/h. The first fraction of 1.5 ml was discarded and the second fraction of 6.5 ml containing androsterone was collected and evaporated to dryness under reduced pressure.

Determination by Enzymatic Cycling Reaction——The dried residue was dissolved in 0.2 ml of ethanol and 20 μl of this sample was poured into each of two mini tubes. Then 100 μl of 3α-HSD solution was added to one tube and 100 μl of Tris–hydrazine buffer containing 0.5 mM NAD^+ was added to the other and both were incubated at 37°C for 10 min. After 30 μl of 1 N NaOH had been added to each tube, the residual NAD^+ was destroyed by heating at 60°C for 15 min. The cycling procedure was followed. First 50 μl of the above reaction mixture and 0.1 ml of 1 mM resazurin solution were added to 1.8 ml of the cycling buffer and the whole was preincubated at 25°C for 3 min. Then 50 μl of the cycling enzyme solution was added to each tube and the initial reaction rate was measured at Ex_{560}, Em_{580} and 25°C. The rate for the blank was subtracted from the experimental rate. For the calculation of androsterone sulfate concentration in serum, the same procedure was carried out using 0.2 ml of androsterone sulfate standard solution in place of the serum.

Reference Analysis——After solvolysis of androsterone sulfate in serum and chromatography of the related steroids on alumina and silica gel, androsterone was quantitated by gas–liquid chromatography according to Rosenfeld and Hellman.[5b] Alumina columns were prepared by pouring a slurry of 4 g of alumina in benzene into a mini column. The sample was dissolved in benzene and applied to the column. The column was washed with benzene, and androsterone was eluted with 0.5% ethanol in benzene. The androsterone obtained from the alumina column was purified by thin-layer chromatography. Samples were

applied to a 20×20 cm plate coated with Silica gel G. The chromatograms were developed in ether. The appropriate bands were scraped from the plate and the steroids were extracted with methanol: chloroform (1: 2). After removal of the solvent, bis(trimethylsilyl)trifluoroacetamide and pyridine were added to each tube, then the tubes were tightly stopped with Teflon plugs and stored overnight at room temperature. The TMS ether solution was analyzed by gas–liquid chromatography (3% OV-17 on Chromosorb W in a 200 cm×3 mm glass column maintained at 180°C; the carrier gas was nitrogen). 5α-Cholestane was used as an internal standard. This method has a coefficient of variation of 6% according to our technique.

Results

Amount of 3α-HSD

We found that 0.2 mU of 3α-HSD for one tube was sufficient to complete the oxidation of 20 μl of 2.9 μg/ml androsterone in ethanol, and the reaction was completed with 10 min at 37°C.

Chromatography of Androsterone on a Sephadex LH-20 Column

According to the method of Habrioux *et al.*, serum containing 0.98 μg/ml of androsterone was solvolyzed and applied to a Sephadex LH-20 column. Androsterone was eluted from 2 to 8 ml.

Destruction of Residual NAD^+

The residual NAD^+ was destroyed almost completely by heating at 60°C for 15 min after the addition of 30 μl of 1 N NaOH. Under these conditions, NADH seemed to be stable, since the recovery was good.

Optimal Conditions for the Enzymatic Cycling Reaction

The optimal temperature and pH of this cycling system were 25°C and pH 8.0, respectively. The cycling frequency was sufficient at about 25 cycle/min (alcohol dehydrogenase 6.25 I.U./tube, diaphorase 4.5 I.U./tube) and increase of either enzyme concentration resulted in a higher blank rate.

Calibration Curve

A linear relationship between androsterone concentration and ΔF/min was obtained in the range 0.035 to 3.5 μg/ml using 20 μl of androsterone in ethanol solution.

Recovery of Androsterone from Serum

The average recovery of 0.7 μg/ml of androsterone sulfate added to ten sera was 97±2.0%.

Reproducibility

The coefficient of variation of the reproducibility of the within-day precision and day-to-day precision using sera were 4.7% (n=10, 0.90 μg/ml androsterone sulfate) and 4.9% (n=7, 0.86 μg/ml androsterone sulfate), respectively.

Effect of 3α-Hydroxysteroids on the Proposed Method

The effect of 3α-OH bile acids, which are the amin 3α-OH steroids in serum, was tested: 0.2 ml of 1 mM cholic acid, deoxycholic acid chenodeoxycholic acid or their glyco- and tauro-conjugates, or lithocholic acid dissolved in the column eluent was applied to a Sephadex LH-20 column and determined by the proposed method.[11] Any recovery of bile acid was less than 0.1% of the initial amount applied to the column. Therefore, the effect of bile acids was negligible. Then the effect of 3α-OH steroid hormones, ethiocholanolone, pregnanediol and androstanediol was also studied. Pregnanediol and androstanediol were eluted later than androsterone on Sephadex LH-20 column chromatography, but etiocholanolone was eluted at almost the same fraction as the androsterone.

Comparison of the Proposed Method with the Conventional Method

In order to compare this method with the gas–chromatographic method, the androsterone sulfate concentrations in 16 sera were determined with this method and the method described

above. As shown in Fig. 2, a good correlation (r=0.993) was obtained between the results obtained by the two methods.

Measurement of Androsterone Sulfate Concentration in Sera of Patients with Hepato-biliary Diseases

The measurements were done with 16 sera. In a few patients (3/16 patients) serum androsterone sulfate concentration was greater than the normal range, but there was no correlation between serum androsterone sulfate concentration and other clinical data.

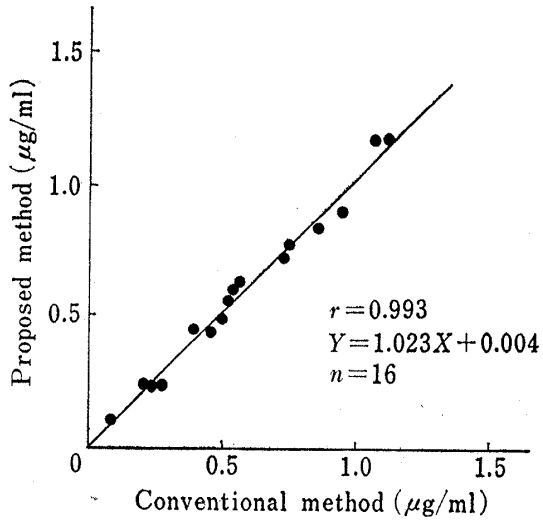

Fig. 2. Correlation of Serum Androsterone Contents Determined by the Proposed Method and the Conventional Method

Discussion

A method for the microassay of serum androsterone sulfate was developed. The conventional analysis of androsterone by gas-liquid chromatography is technically complicated and a large amount of serum is required. The radioimmunoassay which is generally in current use has the drawback that the preparation of a specific antibody is difficult. The advantages of the proposed method are that it is highly sensitive, requires only a small volume of serum, and is simple and rapid. The specificity of the proposed method is strongly dependent on the specificity of 3α-HSD. Indeed, 3α-HSD acts on 3α-OH steroid derivatives as substrates and therefore, androsterone must be extracted prior to the enzyme cycling reaction. In order to separate bile acids which disturb androsterone determination, we adapted the method of Habrioux *et al.*[10] using Sephadex LH-20. The bile acids were not extracted with the Sephadex LH-20 solvent system and androstanediol and pregnanediol were separated from androsterone on Sephadex LH-20 column chromatography. Unfortunately, etiocholanolone was eluted in almost the same fraction as androsterone. Sjövall and Vihko[12] reported that the concentration of solvolyzable etiocholanolone was about 50 times lower than that of androsterone sulfate in plasma, and so the proposed method may not be affected by etiocholanolone. Indeed, the correlation between the proposed method and the gas chromatographic method showed good agreement (r=0.993). The relationship between serum androsterone sulfate concentration and hepato-biliary diseases in relation to androsterone metabolism in the liver, requires further study.

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