Enzymatic Determination of Plasma Dehydroepiandrosterone Sulfate

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SUMMARY A method for determination of Plasma dehydroepiandrosterone sulfate using 3β-hydroxysteroid oxidase is described. The method includes lipides removal and protein precipitation by adding a mixture of chloroform and methanol (2:1, v/v), and solvolysis after extraction with ethylacetate under pH 1 with NaCl saturation, and fluorometric determination using 3β-hydroxy-steroid oxidase, peroxidase and homovanillic acid by the method of G.G. Guilbault et al.

Precision of this method was 5.7% of CV and recovery was 98.2%. These results were satisfactory for determination of plasma dehydroepiandrosterone sulfate in clinical test. An advantage of the method is that the determination can be performed without use of radioactive compound.

Introduction

Studies of conjugated 17-ketosteroids in case of adrenal tumor were reported by E.E. Baulie in 1962 where he insisted the importance of dehydroepiandrosterone sulfate (DHEA-sulfate) and 3β-hydroxy-Δ5-steroids as conjugate in human peripheral venous plasma.

DHEA-sulfate had been determined by gas-liquid chromatography or competitive protein binding assay. Recently, J.E. Buster et al., reported a radioimmunoassay for the direct measurement of DHEA-sulfate in diluted plasma using antiserum reacted with DHEA (1).

We describe here a method for determination of plasma dehydroepiandrosterone sulfate without use of radioactive compounds.

Steps of the procedure are as follows:
I. Plasma → DHEA-sulfate
II. DHEA-sulfate → DHEA
III. DHEA → Androstenedione + H2O2
IV. H2O2 + Homovanillic acid → Flourescent compounds

Materials

Organic solvents and homovanillic acid of analytical grade were used. Peroxidase (EC 1.11.1.7. from horse-radish) was purchased from Boehringer Mannheim,
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dehydroepiandrosterone sulfate (sodium salt) was from Sigma Chemical Co., St. Louis, Mo. 63178 and 3β-hydroxysteroid oxidase (E.C. 1. 1. 3. 6. from B. Sterolicum) was from Kyowa Hakko Co., Ltd., Machida-shi, Tokyo.

The reagent for determination of hydrogen peroxide is that of G. G. Guilbault et al., (2). Phosphate buffer (0.3 mol, pH 7.2), homovanillic acid (2.5 g/liter), horse-radish peroxidase (100,000 U/liter) and 3β-hydroxysteroid oxidase (20,000 U/liter) were used. Standard solution was prepared by dissolving DHEA-sulfate in water (5 µg and 10 µg/ml). A mixture of chloroform and methanol (2:1, v/v) was prepared for the removal of lipides from plasma, as reported by J. Folch et al., (3).

Method

Preparation of sample.

Pipette 1.0 ml of plasma (water as blank and standard) into a 12 ml glass-stoppered test tube, add 6 ml of solvent, chloroform : methanol, 2 : 1 v/v, shake well and add 3 ml of 0.1 mol/liter NaOH solution. Shake 1 min. and centrifuge for 10 min at 2500 rpm. Upper layer of 4 ml is transfered to another test tube and 6 ml of chloroform-methanol solution is added again. Shake well, and centrifuge as described above.

Solvolyis.

Transfer 4 ml of upper layer to a 30 ml tube and adjust to pH 1 with HCl (6 mol/liter) and saturate with 1.5 g NaCl (when the solution is at pH 1, some amount of protein is precipitated.). Shake the solution with 20 ml of ethyl acetate for 5 min. After centrifugation, discard the lower layer and keep the ethyl acetate layer for 10 h at 37°C. Wash the ethyl acetate layer successively with 2 ml of NaOH (80 g/liter) with concde. Na2CO3, and water. After centrifugal separation, transfer 15 ml of ethyl acetate extract to another tube and then evaporate to dryness.

Determination of DHEA.

To the dried residue of ethyl acetate extract, add 2.5 ml of phosphate buffer, 0.1 ml of homovanillic acid solution, 0.2 ml of peroxidase and 0.2 ml of 3β-hydroxysteroid oxidase solution. Incubate for 30 min at 37°C, and then read at 425 nm the intensity of fluorescence excited at 315 nm (HITACHI type 204 Fluorescence Spectrophotomer was used with selector×1 and sensitivity control 11.).

Results

Standard curve. Figure 1 shows a standard curve for DHEA-sulfate (sodium salt) and for DHEA.

Analytical recovery. Recovery of added standard substances to plasma with the

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Fig. 1 A fluorescence intensity (arbitrary units) was measured under condition described in method.

Table 1 Recovery test of DHEA-sulfate added to control serum.

<table>
<thead>
<tr>
<th>added</th>
<th>expected</th>
<th>found</th>
<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>control serum</td>
<td>3.9</td>
<td>3.9</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>5 µg</td>
<td>8.9</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>13.9</td>
<td>100.0</td>
</tr>
</tbody>
</table>

To 1 ml of serum, 50 µl and 100 µl of 100 µg/ml of DHEA-sulfate for the 5 µg and 10 µg addition were added respectively.

Table 2 Effect of added sterols to standard solution of DHEA-sulfate.

<table>
<thead>
<tr>
<th>DHEA-sulfate</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0 µg/ml</td>
</tr>
<tr>
<td>+ 1,000 µg cholesterol</td>
<td>5.1</td>
</tr>
<tr>
<td>+ 100 µg DHEA</td>
<td>4.9</td>
</tr>
<tr>
<td>+ 100 µg pregnenolone</td>
<td>5.2</td>
</tr>
</tbody>
</table>

As free sterols were dissolved in chloroform solution, the additive solution was evaporated to dryness and then 5.0 µg/ml of DHEA-sulfate solution was added and submit the whole procedure.

Table 3 Comparison of DHEA-sulfate values obtained by gas-liquid chromatography and the present method.

<table>
<thead>
<tr>
<th>GLC</th>
<th>µg/ml</th>
<th>Present method</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>4.8</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>0.5</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>2.8</td>
<td>3.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The proposed procedure was 98.2% (mean of 10 samples). Table 1 shows an example of recoveries added to control serum.

Effect of free 3β-hydroxy steroids on DHEA-sulfate assay. As shown in Table 2, all free sterols were removed completely by washing sample with a mixture of chloroform and methanol.

Within-run precision. 10 same samples were assayed and CV was 5.7% with the mean value of 3.95 µg/ml of DHEA-sulfate.

Comparison of normal value. Normal adult values obtained by the present method were 1.5 to 6.0 µg/ml in male and 0.8 to 3.3 in female and those by radioimmunoassay were reported by H. Sekihara et al., as 0.7 to 2.7 µg/ml in male and 0.7 to 2.0 in female (as DHEA-sulfate). Normal adult values obtained by direct radioimmunoassay reported by J. E. Buster et al., were 2.7 to 4.3 µg/ml in man and 1.1 to 4.6 µg/ml for premenopausal and 0.2 to 0.8 µg/ml for postmenopausal in female (1).

Comparison of DHEA-sulfate values by gas-liquid chromatography and the present method was shown in Table 3. Gas-liquid chromatographic determination of DHEA-sulfate in plasma was performed by the method of J. T. France (6).

Discussion

While the sensitive radioimmunoassay has, recently, made it possible to measure steroid hormone in small samples, the present method may be favourable for the laboratories where the isotope labeled compounds can not be used.

There are some unconjugated 3β-hydroxysteroids such as DHEA, pregnenolone and androstenediol in plasma and their concentrations are very low compared with the conjugated 3β-hydroxysteroids. DHEA-sulfate is a principal 3β-hydroxysteroid in plasma, while the 3β-hydroxy steroids such as pregnenolone sulfate are in concentration of 0.1 µg/ml or lower in plasma even at late pregnancy (5). In the 3β-hydroxy-steroid oxidase reaction with unconjugated DHEA, hydrogen peroxide formed can be fairly sensitively measured by the conversion of homovanillic acid to the fluorescent compound in the presence of peroxidase.
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so that the determination of plasma DHEA-sulfate could be performed.

As the sulfate pathway of 3β-hydroxy-Δ5-steroids was proved, DHEA-sulfate was found to be a major adrenal secretory steroid which response to ACTH, and DHEA-sulfate is in equilibrim to unconjugated DHEA and regulates the metabolic conversion rate (7). Plasma DHEA-sulfate, a major adrenal secretory product in man, has been proved to be a valuable tool in assessing adrenocortical function. The present method is, therefore, useful especially for a patient with adrenal tumor which is abnormally increased levels of the DHEA-sulfate in plasma.

References


