Determination of Serum 17α-Hydroxyprogesterone by Liquid Chromatography

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17α-Hydroxyprogesterone, cortisol, and 11-deoxycortisol were extracted with methylene chloride, separated by liquid chromatography, identified by their retention times, and quantitated by monitoring the absorbance at 254 nm. The method is specific and sensitive to as little as 2.5 ng of 17α-hydroxyprogesterone. Mean analytical recovery of added 17α-hydroxyprogesterone was 99% (SD 11%) and the CV for the same or different assays ranged from 3.5 to 9.4%. 6β-Hydroxyprogesterone was used as the internal standard. Concentrations of 17α-hydroxyprogesterone in serum of normal men and women were <2.5 μg/L, but women in the luteal phase of the menstrual cycle had higher values. Mean 17α-hydroxyprogesterone content in serum from a cord blood of normal newborns was 28.1 (SD 13.8) μg/L. Results by this method correlated well (r = 0.97) with results by radioimmunoassay, but were somewhat higher.

Additional Keyphrases: congenital adrenal hyperplasia • comparison with radioimmunoassay • cortisol • 11-deoxycortisol • steroids • newborns • reference interval • values during menstrual cycle • cord blood

Recently, we have developed a liquid-chromatographic method for separating and quantitating cortisol and 11-deoxycortisol in a single serum sample (1). This method has the advantages of being highly specific, simple, and capable of measuring several steroids simultaneously.

17α-Hydroxyprogesterone (17OHP) is an adrenal steroid that is increased in serum of patients with congenital adrenal hyperplasia (2-4), a disease secondary to an enzymic block in the synthesis of cortisol. The measurement of 17OHP is useful in the diagnosis and management of patients with this disorder (5); thus, accurate assays for the quantitation of 17OHP are of great clinical importance. Several methods involving radioimmunoassay (RIA) have been described for the measurement of 17OHP in serum; however, most of them require chromatographic or other purification steps before RIA, making the method cumbersome and resulting in the loss of substantial amounts of 17OHP (6-9).

In these studies, we describe a simple and accurate method in which liquid chromatography is used to measure 17OHP. Cortisol and 11-deoxycortisol can be measured in the same assay. 17OHP was determined in normal humans and patients with congenital adrenal hyperplasia, and the results obtained by liquid chromatography were compared with those obtained by RIA.

Materials and Methods

Analytical methods. The liquid-chromatographic method used to measure steroids in serum has been described in detail previously (1): 1 mL of serum was extracted with methylene chloride and the extract was washed with sodium hydroxide (0.1 mol/L) and water, dried, and dissolved in methanol/water (55/45 or 60/40, by vol) before analysis by liquid chromatography. The steroids used for standards (obtained from Sigma Chemical Co., St. Louis, MO 63178, and Steraloids, Inc., Wilton, NH 03086) were dissolved in methanol/water. We used a Model 204 liquid chromatograph (Waters Associates, Inc., Milford, MA 01757) equipped with a μBondapak C18 column. The wavelength used was 254 nm, the sensitivity 0.005 or 0.01 absorbance unit full scale, and the flow rate of the mobile phase (methanol/water, 55/45 or 60/40, by vol) was 1 mL/min.

Serum 17OHP was determined by RIA at Laboratory Procedures, Inc., King of Prussia, PA 19406; at Bio-Science Laboratories, Van Nuys, CA 91405; and at the Nicholas Institute, San Pedro, CA 90731, in each case by modifications of established methods (7, 8).

Human subjects. Venous blood samples were obtained from 30 normal adults—seven men and 23 women (nine in follicular and nine in luteal phase of their menstrual cycle and five post-menopausal)—and from six patients with congenital adrenal hyperplasia. These patients were evaluated clinically and with appropriate tests and found to have 21-hydroxylase deficiency of the salt-losing (two patients) and nonsalt-losing (four patients) varieties. Patients were studied before and during treatment with cortisol acetate or prednisone and, if needed, fludrocortisone acetate. Mixed arterial and venous umbilical cord blood was obtained from 28 normal newborns and from one with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Serum was separated by centrifugation and stored at −20 °C until assay.

Statistical methods. Correlation between liquid chromatography and RIA was tested by linear correlation, and significance of differences between assays was assessed by paired t-test.

Results

17OHP was separated by liquid chromatography, identified by its retention time, and quantitated by the height of the chromatographic peak. The retention time varied with the concentration of methanol used in the mobile phase; at a proportion (by vol) of 55/45 (methanol/water) it was 32.5 min, and at 60/40 it was 21 min.

Specificity. 17OHP was isolated and accurately quantitated in 139 of 143 serum samples tested; in four samples, an additional chromatographic peak interfered with its measurement. 17OHP was separated from cortisol, 11-deoxycortisol, and the internal standard, 6β-hydroxyprogesterone, their respective retention times (in methanol/water, 55/45, by vol) being 10, 17, and 24 min (Figures 1 and 2).

Sensitivity. Amounts of 17OHP as low as 2.5 ng could be quantitated and the standard curve was linear up to a concentration of 160 ng (data not shown). 17OHP concentrations as low as 2.5 μg/L could be measured in 1 mL of human serum.

Analytical recovery. The percent recovery was determined by adding 200 μg of 17OHP per liter to 53 serum samples containing endogenous 17OHP ranging from 0 to 233.3 μg/L.
The mean recovery was 99(SD 11)% and it was not affected by the concentration of endogenous 17OHP. Percent recovery was also determined by adding 3, 10, 30, 100, or 300 μg of 17OHP per liter to five different aliquots of a serum pool containing no detectable 17OHP; the respective results (n = 9 to 13) were 105(SD 7)%), 101(SD 6)%), 95(SD 7)%), 105(SD 6)%), and 102(SD 8)%.

Precision and accuracy. Within-assay and between-assay variability were assessed in sample pools containing 3, 10, 30, 100, or 300 μg of 17OHP per liter added to a serum pool containing no detectable 17OHP. CV ranged from 3.9 to 9.4% in the same assay and from 3.5 to 8.3% in different assays (Table 1).

Internal standard. 6β-Hydroxyprogesterone, a steroid not found in serum but having physical and chemical characteristics similar to those of the steroids studied, was used as internal standard for this assay. Its retention time was close to that of cortisol, 11-deoxycortisol, and 17OHP (Figure 1), and we were able to isolate and quantitate it in 183 of 187 serum samples to which it had been added before the extraction procedure.

Human studies. The respective concentrations of 17OHP in serum of normal men and post-menopausal women were less than 2 μg/L and 1 μg/L, and the values in the follicular and luteal phases of the menstrual cycle were 0 to 2.5 μg/L and 0 to 3.3 μg/L, respectively. Nineteen serum samples from six treated and untreated patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency contained 17OHP concentrations ranging from 0 to 233.3 μg/L. Mean 17OHP concentrations in serum obtained from mixed cord blood of normal newborns were 28.1 (SD 13.8) μg/L; in one sample obtained from a baby with 21-hydroxylase deficiency, the value was 118.8 μg/L.

Comparison with RIA

Twenty-three serum samples from normal persons and patients with congenital adrenal hyperplasia were measured for 17OHP content by liquid chromatography and RIA. While there was good correlation between results by the two assays (r = 0.97), the values obtained by liquid chromatography were somewhat higher than those obtained by RIA (p < 0.05; Figure 3). When known amounts of 17OHP were added to a serum sample containing no 17OHP, the values obtained by liquid chromatography compared well with those of three RIA procedures tested (Table 2).

Discussion

In this liquid-chromatographic method for isolating and quantitating 17OHP, we were able to separate 17OHP from cortisol and 11-deoxycortisol in the same serum sample. The assay was specific for 17OHP, we could accurately measure concentrations as low as 2.5 μg/L, the recovery of added 17OHP was about 100%, and the assay variability was less than 10%. In only four out of 143 samples, 17OHP could not be quantified accurately, owing to the presence of an interfering chromatographic peak. 6β-Hydroxyprogesterone was found satisfactory as internal standard.

Concentrations of 17OHP in serum of normal men and women, post-menopausal or in follicular phase of their menstrual cycle, were less than 2.5 μg/L; somewhat higher values

![Fig. 1. Chromatogram obtained on injection of cortisol (F, 60 ng), 11-deoxycortisol (S, 60 ng), 6β-Hydroxyprogesterone (6β OHP, 120 ng), and 17α-hydroxyprogesterone (17α OHP, 60 ng) standards in methanol/water, 55/45 by vol](image)

![Fig. 2. Chromatogram of extract of serum from a patient with congenital adrenal hyperplasia demonstrating cortisol (F) and 17α-hydroxyprogesterone (17α OHP) peaks](image)

**Table 1. Variability of 17α-Hydroxyprogesterone Concentrations by Liquid Chromatography**

<table>
<thead>
<tr>
<th>17OHP added, μg/L</th>
<th>Within assay</th>
<th>Between assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 17OHP (and SD), μg/L</td>
<td>CV, %</td>
<td>Mean 17OHP (and SD), μg/L</td>
</tr>
<tr>
<td>3</td>
<td>3.2 (0.3)</td>
<td>9.4</td>
</tr>
<tr>
<td>10</td>
<td>10.3 (0.4)</td>
<td>3.9</td>
</tr>
<tr>
<td>30</td>
<td>29.6 (2.3)</td>
<td>7.8</td>
</tr>
<tr>
<td>100</td>
<td>105.9 (6.5)</td>
<td>6.1</td>
</tr>
<tr>
<td>300</td>
<td>296.3 (20.1)</td>
<td>6.8</td>
</tr>
</tbody>
</table>

*Mean and SD of replicate analyses, five or six determinations performed in the same assay or five to six determinations performed in a different assay.*
were obtained for samples collected during the luteal phase. Values for 17OHP in mixed venous and arterial cord blood varied; the mean value was about 10-fold that for adults. This may have been partly related to increased cortisol-binding globulin to which 17OHP also is known to be bound (10). Results obtained for adults and newborns are similar to those reported by other investigators, who used RIA (6-9, 11). In one case with congenital adrenal hyperplasia, we found 17OHP concentrations in cord blood to be about fivefold higher than normal, suggesting that cord blood analysis for 17OHP may be of value in early diagnosis of the disease. However, further studies in this area are necessary.

Although values for 17OHP as obtained by liquid chromatography were somewhat higher than by RIA, results by the two assays correlated well. RIA is a more sensitive method for 17OHP than liquid chromatography, but future developments such as new detectors and the use of gradient systems for the mobile phase should increase the sensitivity of liquid chromatography. The advantages that liquid chromatography offers over other current methods for measurement of 17OHP are its ability to separate and measure the steroid in a single step, its great specificity, and the complete analytical recovery of added 17OHP. In addition, the method is simple, accurate, and capable of also measuring cortisol and 11-deoxycorticisol in the same assay. These other determinations should allow for a better tool in the diagnosis and management of patients with various forms of congenital adrenal hyperplasia. In conclusion, these studies indicate liquid chromatography to be a useful method for measuring 17OHP in addition to cortisol and 11-deoxycorticisol. As new refinements of the assay become available, more steroids could be measured simultaneously in a single serum extract, thus increasing the capability, efficiency, and clinical applications of the method.

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References

Table 2. Comparison of Liquid Chromatography (LC) and Three Radioimmunoassay Methods for 17\(\alpha\)-Hydroxyprogesterone

<table>
<thead>
<tr>
<th>17OHP added, (\mu g/L)</th>
<th>LC</th>
<th>RIA A</th>
<th>RIA B</th>
<th>RIA C</th>
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<tbody>
<tr>
<td>0</td>
<td>&lt;0.3</td>
<td>0.5</td>
<td>0.4</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td>3.9</td>
<td>2.6</td>
<td>4.2</td>
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<tr>
<td>10</td>
<td>10.7</td>
<td>7.7</td>
<td>11.0</td>
<td>9.7</td>
</tr>
<tr>
<td>30</td>
<td>26.7</td>
<td>40.4</td>
<td>28.0</td>
<td>31.0</td>
</tr>
<tr>
<td>100</td>
<td>97.3</td>
<td>66.7</td>
<td>71.0</td>
<td>125.0</td>
</tr>
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</table>

* In a serum sample that contained no detectable 17OHP, before and after addition of the steroid.