Simultaneous Measurement of Eight Corticosteroids by Liquid Chromatography, and Application of the Procedure to Diagnosis of Congenital Adrenal Hyperplasia

Wei Ji-qing, Zhou Xian-fang, and Wei Ji-lu

We describe a liquid-chromatographic procedure for simultaneously determining eight steroids in serum. We used a Zorbax ODS column and a mobile phase of methanol/isopropanol/water (44/10/46, by vol), which well resolves the steroids cortisol, cortisone, 11-deoxycorticosterone, 11-deoxy cortisol, 11-deoxycortisosterone, androstenedione, 17-hydroxyprogesterone, and progesterone, but not 11-deoxycorticosterone and androstenedione. Analytical recoveries of the steroids ranged from 89.27% to 99.58%. CVs were <10%. Prednisone and dexamethasone do not interfere. Using this method, we studied the serum steroid profiles of six patients with congenital adrenal hyperplasia (CAH) due to deficiency of 21-hydroxylase or 17-hydroxylase. Not only could we make a clearcut diagnosis and distinguish the subtle types of CAH, but also we could investigate clinical and biochemical variants of CAH. For example, we confirmed 17-hydroxylase deficiency in a patient who was normotensive with primary amenorrhea and streak gonads.

Additional Keyphrases: 17- and 21-hydroxylase deficiency steroids distinguishing types of congenital adrenal hyperplasia reversed-phase "high-performance" chromatography RIA compared reference interval urine

Assay of some steroids in serum and urine is necessary for biochemical diagnosis and classification and for monitoring therapy of congenital adrenal hyperplasia (CAH). Present, steroids are commonly measured by the competitive protein-binding technique, radioimmunoassay (RIA), or gas chromatography. However, the competitive protein-binding method lacks specificity, and the steroid being measured must be the principal corticosteroid present in serum if results are to be reliable (1, 2). The RIA is still burdened with a variety of methodological problems (3–5, 18). The antisteroid antibodies used can cross react to varying degrees with analogs of the steroid assayed (6–9). Lack of specificity of the direct RIA especially detracts from correct evaluation of CAH, because patients with CAH have a large variety of steroids in their serum. It is often necessary to use chromatographic or other purification steps before RIA. Moreover, to obtain a steroid profile and ascertain ratios of some steroids in serum, use of multiple RIA systems is required, because each RIA can determine only one, or one type, of steroid. This is not only time- and cost-consuming, but also may not be valid because of between-assay and between-laboratory variation (3). Gas chromatography can be used to measure many steroids in single assay, but the derivatization techniques involved are tedious and require considerable technical skill (10, 11) and steroids with several functional groups can give a mixture of derivatives so that a purification step prior to injection often is required (10), which is also time-consuming. Under these circumstances, a simpler and more specific way of obtaining the steroid profile of serum is highly desirable.

Recent work in several laboratories (12–27) has shown that "high-performance" liquid chromatography can be used to determine the concentration of steroids in biological samples, but only a few such assays have involved simultaneous measurement of various steroids and applications to clinical practice. This paper deals with a modified isocratic reversed-phase liquid-chromatographic procedure for measuring serum cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycorticosterone (DOC), androstenedione (A), 17α-hydroxyprogesterone (17-OHP), and progesterone simultaneously, and a study of the steroid profiles in patients with CAH by this method.

Materials and Methods

Apparatus and Chromatographic Conditions

We used a Model LC-3A liquid chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a 25 cm × 4.6 mm (i.d.) Zorbax ODS column. Absorbance was measured at 240 nm, with the sensitivity set at 0.01 A full scale. The mobile phase was methanol/isopropanol/water (44/10/46, by vol), which was pumped at a flow rate of 0.7 mL/min. Column temperature was 55°C.

Preparation of Sample Solutions

Serum samples were stored at −30°C until assay. We extracted 2 mL of serum with 10 mL of methylene chloride, washed the extract with 3 mL of 0.1 mol/L NaOH and then with 3 mL of water, and evaporated the solvent in a water bath at 40–45°C. We dissolved the residue in 50 μL of mobile phase with use of an ultrasonic bath for 2 min, and then injected 25 μL of the supernate into the chromatograph.

Comparison with RIA

For comparison, we also measured the concentrations of cortisol in serum with a 125I-labeled cortisol RIA kit supplied by Beijing Chemical Factory, Beijing, China. These sera were not purified by chromatographic steps before the RIA.

Clinical Data

Subjects. We studied 30 normal subjects, 15 men and 15 women, ages 19 to 50 years, as our control group.

We studied six patients with CAH, ages 6 to 24 years. Four of them were diagnosed as having a simple virilizing type of 21-hydroxylase deficiency, on the basis of male
pseudoprecocious puberty (patient no. 1) or female pseudohermaphroditism (patients 2, 3, and 4), and above-normal concentrations of 17-ketosteroids with normal concentrations of 17-hydroxycorticoids in urine. Three patients (no. 1, 2, and 3) were untreated, the fourth had been treated with 15 mg of prednisone per day for longer than one year and had menstruated. The other two patients (no. 5 and 6, women) were siblings of 46 XX genotype with 17-hydroxylase deficiency. One (no. 5), 24 years old, was hospitalized for hypertension (180/120 mmHg), hypokalemia, primary amenorrhea, and sexual infantilism. During treatment with dexamethasone (0.375 mg per day), her blood pressure returned to the normal value (120/80 mmHg), but after the cessation of glucocorticoid therapy, it increased again. The other (no. 6), 19 years old, had not menstruated, nor had secondary sex characteristics developed, but she was normotensive (100/70 mmHg). Both of these patients showed streak gonads by pneumoperitoneography.

Study Maneuvers

**Basal values:** Blood samples used for measuring steroids were obtained by venipuncture between 0800 and 0900 h.

**Response to corticotropin stimulation:** Three normal women and one patient (no. 6) were injected intramuscularly with 25 int. units of bovine corticotropin at 0800 h; blood was sampled immediately before the injection and 60 min afterwards.

**Response to dexamethasone suppression:** One patient (no. 5) ingested 0.75 mg of dexamethasone every 8 h for two days. Blood was sampled from her at 0800 h on the third morning. Another patient (no. 6) was given a single dose of dexamethasone (0.75 mg, orally) at 2300 h; her blood was sampled at 0800 h the next morning.

Statistical Methods

Correlations between the concentrations of corticosteroid standards and the peak areas recorded by a data processor were analyzed by the least-squares method. Concentrations of cortisol measured by the HPLC system and RIA were compared by Student's two-tailed t-test. Unpaired data were compared by use of the Wilcoxon rank sum test. All P values were two-tailed. Significance was defined as P < 0.05. Reference intervals were calculated by the method of percentiles and expressed as a median and 80% confidence limits, because the data were not gaussian distributed.

Results

General

Figure 1 shows a typical elution profile of standard steroids. The eight steroids were well resolved except for A and DOC. The chromatographic retention time (min, mean ±SD, n = 30) for cortisone was 6.54 ± 0.04, for cortisol 7.10 ± 0.03, for corticosterone 10.11 ± 0.05, for 11-deoxycortisol 10.58 ± 0.03, for A and DOC 17.09 ± 0.12, for 17-OHP 19.54 ± 0.09, and for progesterone 45.09 ± 0.33 (CVs ranged from 0.32% to 0.74%). The eight steroids in serum were identified on the basis of their retention times (mean ±SD). Regression equations were calculated from logarithms of peak areas and concentrations of these steroids in standard mixtures containing 5 to 500 μg each of cortisol, cortisone, corticosterone, 11-deoxycortisol, DOC, 17-OHP, and progesterone per liter. Good linear relationships were obtained (r >0.99, P < 0.0005). All the standard curves were linear up to a concentration of 500 μg/L. The steroids in serum were quantified by comparison with external standards.

Accuracy

Analytical accuracy was assessed by adding 200 ng of cortisol, 50 ng of corticosterone, and 25 ng each of 11-deoxycortisol, DOC, 17-OHP, and progesterone to 2-mL portions of normal serum. Five replicates were measured by the HPLC method. Mean (±SD) analytical recoveries, in percent, were 89.27 ± 7.95 for cortisol, 94.01 ± 9.14 for corticosterone, 97.78 ± 9.64 for 11-deoxycortisol, 99.58 ± 1.14 for DOC, 98.58 ± 5.03 for 17-OHP, and 90.02 ± 2.34 for progesterone.

Precision

The precision of the HPLC method was examined by measuring a normal serum and a mixture of the reported steroid standards. Four 2-mL samples taken from the serum pool were measured daily for three days (Table 1). Four 2-mL samples taken from the standard mixture were measured on four different days (Table 2). The results show that estimates of the steroids in each pool did not differ significantly (P > 0.05) on any given day (intra-assay variance) or from day to day (interassay variance). Intra-assay CVs ranged from 1.46% to 4.89%, interassay CVs from 2.12% to 7.73%.

Sensitivity

Detection limits (a signal/noise ratio >2) for the present method for these steroids in serum were all 0.5 μg/L, except for progesterone, for which the limit was 2.5 μg/L.

Reference Interval

In serum from the normal subjects, the median concentration of cortisol was 80 μg/L (normal range: 50–160 μg/L),
Table 1. Evaluation of Precision in the HPLC Analysis of Steroids in Serum

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Concn of steroids, µg/L</th>
<th>Intra-assay CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD, n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>cortisol</td>
<td>22.54 ± 0.62</td>
<td>2.05–4.80</td>
</tr>
<tr>
<td>cortisone</td>
<td>97.60 ± 3.47</td>
<td>1.46–4.22</td>
</tr>
<tr>
<td>progesterone</td>
<td>8.55 ± 0.38</td>
<td>4.13–4.46</td>
</tr>
</tbody>
</table>

The concentration of other steroids being extremely low or undetectable. The median concentration of cortisol was 16 µg/L (normal range 6–24 µg/L); of corticosterone was <0.5 µg/L (normal range: 0–1.8 µg/L); of 11-deoxycortisol was <0.5 µg/L (normal range: 0–1.0 µg/L); of A + DOC was 1.3 µg/L (normal range: 0–3.8 µg/L); of 17-OHP in men was 6 µg/L (normal range: 0–8.5 µg/L) and in women was <0.5 µg/L (normal range: 0–1.4 µg/L); and of progesterone was <2.5 µg/L (normal range: 0–2.5 µg/L), except for premenstrual women, for whom the median was 6 µg/L with a range of 3.3 to 11 µg/L (n = 5).

Three hydroxylase indexes were calculated by the following formulas:

21-Hydroxylase (EC 1.14.99.10) index =

\[
\frac{\text{cortisone (µg/L)} + \text{cortisol (µg/L)} + \text{deoxycortisol (µg/L)}}{17-\text{OHP (µg/L)}}
\]

11β-Hydroxylase (EC 1.14.15.4) index =

\[
\frac{\text{deoxycortisol (µg/L)}}{\text{cortisone (µg/L)} + \text{cortisol (µg/L)}}
\]

17α-Hydroxylase (EC 1.14.99.9) index =

\[
\frac{\text{cortistone (µg/L)}}{\text{cortisone (µg/L)} + \text{cortisol (µg/L)} + 17-\text{OHP (µg/L)}}
\]

For the control group these respective indexes ranged from 0 to 0.20 with a median of 0.02, from 0 to 0.15 with a median of 0, and from 0 to 0.12 with a median of 0.

After administration of corticotropin, the concentration of cortisol in serum from three normal women was increased significantly (P < 0.05, Figure 2).

In serum from untreated 21-hydroxylase-deficient patients (no. 1, 2, and 3) the basal concentrations of 17-OHP and of A + DOC were considerably higher than in the normal control group (P < 0.01), and concentrations of cortisone and cortisol were markedly lower than in the control group (P < 0.01). In addition, the concentration of progesterone in patient no. 1 was increased. The 21-hydroxylase index of all three patients was significantly greater than that of the control group (P < 0.01), 25-fold the upper limit of normal (Table 3). The chromatograms of these patients showed a stair-like "tri-peak," composed of peaks for A + DOC, 17-OHP, and an unknown eluting between 17-OHP and A + DOC, as well as two other abnormal unidentified peaks with retention times of 9.49 min and 10.86 min, respectively (Figure 3). In the treated 21-hydroxylase-deficient patient (no. 4), the concentrations of cortisone and cortisol in serum were also below the normal range, but concentrations of 17-OHP and A + DOC were within it (Table 3), and there was no "tri-peak" in her chromatogram (Figure 4).

In the patient with classical 17-hydroxylase deficiency (no. 5), the basal concentrations of cortisone and cortisol were below the normal range, that of corticosterone was extremely high, and the value for A + DOC was slightly above normal. Her 17-hydroxylase index was as high as 7.39, and the peak for corticosterone in her chromatogram was quite high (Figure 5). After administration of dexamethasone, the peak disappeared. In patient no. 6, who had 17-hydroxylase deficiency, but not the classical form, her basal profile for serum steroids was similar to that for her sister, except that serum A + DOC were normal for no. 6 (Table 3 and Figure 6). After administration of corticotropin, the concentration of corticosterone in her serum increased to twice the basal value; however, the concentrations of cortisone and cortisol did not increase. After the dexamethasone suppression test, the increased value of corticosterone decreased dramatically towards the normal value.

Comparison with RIA

Twenty-three serum samples from the 20 normal subjects

![Graph showing chromatograms of serum from a normal woman before (left) and after (right) administration of corticotropin. Peaks: A, cortisone; B, cortisol; C, corticosterone; E, A + DOC; F, 17-OHP; G, progesterone. Retention time (in min) indicated for major peaks.](image)

Table 2. Evaluation of Precision in the HPLC Analysis of Seven Steroids in a Mixture of Standards

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Concen, µg/L</th>
<th>Interassay CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD, n = 4)</td>
<td></td>
</tr>
<tr>
<td>cortisol</td>
<td>99.73 ± 3.54</td>
<td>3.55</td>
</tr>
<tr>
<td>cortisone</td>
<td>506.46 ± 26.30</td>
<td>5.19</td>
</tr>
<tr>
<td>corticosterone</td>
<td>96.08 ± 3.34</td>
<td>3.50</td>
</tr>
<tr>
<td>deoxycortisol</td>
<td>103.27 ± 4.40</td>
<td>4.27</td>
</tr>
<tr>
<td>DOC</td>
<td>126.30 ± 2.75</td>
<td>2.18</td>
</tr>
<tr>
<td>17-OHP</td>
<td>105.08 ± 2.24</td>
<td>2.13</td>
</tr>
<tr>
<td>progesterone</td>
<td>49.67 ± 3.84</td>
<td>7.73</td>
</tr>
</tbody>
</table>
and six serum samples from the six patients with CAH were measured for cortisol by the present method and by the direct RIA. In the normal group, the concentrations of cortisol measured by HPLC were not significantly different from those measured by RIA (P >0.05, n = 23). The correlation coefficient between the two assays was 0.89 (n = 23, P <0.001) with a slope of 0.92 and a y-intercept of 1.12 μg/L. However, the basal concentrations of cortisol as measured by RIA in patients no. 1, 2, 3, 4, 5, and 6 were 230, 190, 250, 80, 36, and 20 μg/L, respectively, significantly higher than those by HPLC (P <0.02). The concentrations of cortisol in patients with 21-hydroxylase deficiency were even higher than that of the upper confidence limit (median: 9 μg/L, reference interval: 43.6 to 160 μg/L, n = 20, by RIA).

Discussion

We have described here an HPLC procedure involving reversed-phase chromatography for separating and measuring steroids in serum. Slight modification of the present method allows assay of more steroids in the same sample than do previously described methods (12-27). It offers improved efficiency, capability, and clinical application. The HPLC method provides great specificity, which can circumvent the pitfalls ofRIA antibody cross reactivity. This is very important for correct evaluation of CAH. Patients with CAH have a large variety of steroids in their serum, many of which are quite similar, such as cortisol and 21-deoxycortisol (28-30). When significant quantities of 21-deoxycorticosteroid and other unknown steroids are present in serum, comparatively nonspecific methods such as the competitive protein-
binding technique and RIA may give false-positive values for cortisol (18, 31). We encountered three patients with untreated 21-hydroxylase deficiency who had an increased concentration of cortisol in serum as assayed by a direct RIA. This may be due to cross reactivity of the antibody with 21-deoxycortisol or other unidentified steroids, too. However, these interferences, as well as those by some drugs such as prednisone and dexamethasone, could not disturb measurement of the steroids by the present method. In addition, this method is simple, reproducible with respect to retention times and quantitative evaluation, easy to perform even by inexperienced personnel, and time- and cost-saving. With this method, we not only can reliably measure the concentration of each steroid, to give a steroid "profile," but we also can obtain valid ratios between precursors and products in the key steps of steroidogenesis, which are obtained with difficulty by other methods.

The practical significance of this method has preliminarily been shown through the clinical studies in six patients with CAH. By applying this method, it apparently is easy to make a clearcut diagnosis and distinguish subtle types of classical CAH. The characteristics of the serum profile in a simple virilizing type of 21-hydroxylase deficiency were as follows: basal concentrations of cortisol and cortisone were extremely low, whereas those of A + DOC and 17-OHP, as well as the 21-hydroxylase index, were very high. There was a stair-like "tri-peak" in their chromatograms. Our chromatogram differs slightly from the chromatograms of a patient with 21-hydroxylase deficiency provided by Canalis et al. (20). In that chromatogram there was a clear peak for "cortisol" and a broad, smooth, solitary peak for "17-OHP" instead of the "tri-peak." We suspect that such a chromatogram resulted from the separation efficiency, which was not enough to resolve cortisol and 17-OHP from some other steroids such as the unidentified compounds for which retention times were 9.49 min and 18.61 min, respectively, in our patients' chromatograms (Figure 3). On the other hand, the chromatogram maybe came from a patient with partial 21-hydroxylase deficiency. The characteristics in 17-hydroxylase deficiency were below-normal basal concentration of cortisone and cortisol in serum with markedly increased corticosterone concentration and 17-hydroxylase index. Although up to now no serum from a patient with 11- or 18-hydroxylase deficiency has been available to us, the characteristic serum steroid profile in these disorders is predictable according to reference 32 and steroidogenesis theory. It appears that the diagnosis of classical CAH can be established without dynamic tests of the pituitary–adrenal axis, because the changes in the serum steroid profile for CAH mentioned above were significantly different from those for normal subjects and we found that they did not overlap between groups. This method can also be used to investigate clinical and biochemical variation of CAH. Some patients with special features have been reported, e.g., with late- or adult-onset 21-hydroxylase deficiency (33), partial 21-hydroxylase deficiency (34), or variant types of 11-hydroxylase deficiency (absence of DOC or little impairment of cortisol synthesis, or without hypertension) (35). Such patients are difficult to identify with other routine methods, but very easy to identify with the present method. For example, as reported here, we diagnosed an interesting case of 17-hydroxylase deficiency (patient no. 6) without hypertension and similar to pure gonadal dysgenesis. To our knowledge, such a 17-hydroxylase deficiency has never been reported before.

We also use the method to monitor therapy of CAH. We found that changes in the serum steroid profile for patients with treated 21-hydroxylase deficiency correlated with their clinical response to therapy. Satisfactory markers of optimal therapeutic control of various enzymatic defects remain to be investigated. Finally, the chromatogram contained some abnormally high peaks for unidentified compounds, which prompted us to further studies.

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References

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