Assay of Plasma Testosterone During the First Six Months of Life: Importance of Chromatographic Purification of Steroids

John S. Fuqua, Ellen S. Sher, Claude J. Migeon, and Gary D. Berkovitz

Determination of the plasma concentration of testosterone (T) is important in evaluating infants born with ambiguous genitalia and micropenis, and several commercially available kits provide a direct assay of T in unextracted plasma. Using plasma samples obtained from 36 subjects <6 months old, we compared the concentration of plasma T measured by RIA after extraction and purification by column chromatography with the T concentration measured in a direct assay. When aliquots of samples were purified before RIA, the concentration of T was markedly lower than in the direct assay. In the first 3 weeks postpartum, results of the direct assay were 3.8-fold greater than those obtained after purification. This difference decreased over time, and by age 2 months there was fairly good agreement between the two methods. These data indicate that some direct assays of plasma T are inappropriate during the first 2 months postpartum.

**Materials and Methods**

**Patient Population**

Plasma samples were obtained from 36 subjects (22 males and 14 females) at ages 1 day to 5 months. Three samples were obtained from each of two male infants and a single sample from each of the remaining 34 subjects. Approximately one-half of the subjects were ascertained at the Pediatric Endocrine Clinic of the Johns Hopkins Hospital to have various disorders of sexual differentiation, including congenital adrenal hyperplasia, 46,XY gonadal dysgenesis, 45,X/46,XY mosaicism, and isolated micropenis. The remaining subjects were ascertained anonymously from those undergoing karyotype analysis at the Genetics Laboratory of the Kennedy Krieger Institute. All samples were obtained in accordance with the guidelines of the Institutional Review Board of the Johns Hopkins University School of Medicine. Three specific cases of ambiguous genitalia are presented to illustrate the importance of accurate determination of plasma T.

Case 1, born at 30 weeks' gestation, had a karyotype of 46,XY. The phallus was only 13 mm long (normal 18–32 mm) (6), and the urethral meatus was at the base. The labioscrotal folds were fused. The right gonad was palpable in the labioscrotal fold, and the left gonad was palpable in the inguinal canal. Because of the baby's prematurity, a genitourethrogram was not performed.

Case 2 was a term infant with karyotype 45,X/46,XY. The phallus was 3.0 cm long, and there was perineal hypospadias. Gonads were palpable in the inguinal canals bilaterally. A genitourethrogram and a sonogram identified a large utriculovaginal pouch and a rudimentary uterus.

Case 3 was born at 36 weeks' gestation and had mild clitoromegaly. The karyotype was 46,XX. There was no posterior labial fusion, and no gonads were palpable. Ultrasonography indicated the presence of a normal uterus.

**Radioimmunoassay**

Specimens were stored at −20 °C until assayed. Samples were divided into aliquots for direct assay of T

**Indexing Terms:** neonates/sex differentiation disorders/cryptorchidism/radioimmunoassay/intemethod comparison/sample preparation

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Table 2. Plasma testosterone concentrations measured in three patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Without extraction &amp; purification</th>
<th>With extraction &amp; purification</th>
<th>Normal range for age*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 weeks</td>
<td>7.42</td>
<td>2.84</td>
<td>5.21–10.42 (male)</td>
</tr>
<tr>
<td>2</td>
<td>2 days</td>
<td>6.21</td>
<td>1.80</td>
<td>2.08–7.12 (male)</td>
</tr>
<tr>
<td>3</td>
<td>1 day</td>
<td>8.81</td>
<td>1.56</td>
<td>0.97–2.08 (female)</td>
</tr>
</tbody>
</table>

* Source: ref. 7.

Table 2 shows the plasma T concentrations of subjects 1–3 determined by direct assay and after chromatographic purification. The plasma concentrations of T determined by direct assay in patients 1 and 2 were similar to those of normal newborn males. By contrast, the results determined after chromatographic purification were abnormally low. The plasma concentration of T after direct assay in patient 3 was abnormally higher than that of a normal newborn female, but the concentration after purification was normal.

Figure 1 portrays the relationship between the concentration of T determined by the two methods in the first 3 weeks postpartum and from ages 3 weeks to 5 months. In almost all cases, the direct method yielded spuriously higher results in the first 3 weeks, the results in this age group averaging 3.8-fold higher than those obtained after purification of steroid. However, the difference between the results of the two assays decreased over time, so that after age 3 weeks (Fig. 1, right) there was fairly good agreement between the two methods. In only 6 of the 40 samples were the values obtained after purification higher than those obtained by the direct RIA alone.

Results of experiments performed to characterize the interfering substance(s) are summarized in Table 3. Apparently, the interfering material remains in the plasma after CCl₄ extraction. In particular, the concentrations of T determined after column chromatography are similar to those determined after extraction alone. The results of direct RIA of the eluate from the column showed only a single peak, in the fraction corresponding to that in which the [³H]T eluted (data not shown).

Discussion

Sex differentiation is considered to consist of several interrelated steps: determination of genetic sex; formation of undifferentiated, bipotential structures; and determination of gonadal sex, which results in differentiation of external genitalia and internal sex ducts (7). Incomplete masculinization in a 46,XY fetus can result from abnormalities at any one of the steps of male sex differentiation. In particular, incomplete masculinization is caused by either insufficient T production or insufficient androgen effect despite normal T production. This observation underscores the importance of the accurate determination of T concentration.

Inappropriate masculinization of an infant with a
46,XX karyotype may result from a virilizing form of adrenal hyperplasia, excess maternal androgen, and other rare abnormalities.

For evaluation of patients with ambiguous genitalia, we have elsewhere proposed a scheme (8) designed to provide information for diagnosis, treatment, and gender assignment. In the course of this evaluation, the plasma concentration of T is determined, ideally within the first 2 days postpartum.

An abnormally low concentration of plasma T in an infant with a 46,XY karyotype suggests an abnormality of gonadal differentiation or T biosynthesis such that testosterone production may be subnormal at puberty. By contrast, a normal concentration of plasma T in an infant with ambiguous genitalia indicates the possibility of an abnormality of androgen action. Similarly, abnormally high concentrations of plasma T in newborn infants with 46,XX karyotypes raise the possibility of an abnormal source of androgen.

Because of some concern about the accuracy of the direct T assay without extraction and purification, we compared the results of T obtained by either a direct assay or an assay including extraction and chromatographic steps. (Note that, according to the manufacturer, the RSL 125I Testosterone Assay is not designed for use in newborns.) As presented here, we found that the measured plasma T concentrations may be spurious in the first 3 weeks postpartum unless the steroid is purified before assay. Given the relatively small number of samples studied, however, there may also be some infants in the older age group for whom direct assay of T concentration yields inaccurate results.

Cases 1–3 illustrate potential pitfalls of the laboratory evaluation. The falsely high concentrations of plasma T in 46,XY (case 1) or 45,X/46,XY individuals (case 2) could have produced problems in sex assignment by creating false expectations of future gonadal function. Moreover, unnecessary surgical procedures might have been performed in a 46,XX infant with abnormally high plasma T (e.g., case 3) to investigate and remove presumed testicular tissue.

The cause of the spurious increase of plasma T is unknown but may be related to high concentrations of

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Table 3. Effect of extraction and purification on individual plasma testosterone concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Plasma testosterone conc, nmol/L</th>
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<tbody>
<tr>
<td></td>
<td>Without extraction and purification</td>
</tr>
<tr>
<td>1</td>
<td>7.19</td>
</tr>
<tr>
<td>2</td>
<td>7.53</td>
</tr>
</tbody>
</table>
38-hydroxy-Δ4-steroid sulfates, which influence the assay of 17-hydroxyprogesterone in the newborn period (5). The results of our experiments characterizing the cross-reacting substance in the infants' plasma are consistent with this possibility, because relatively polar compounds such as these would not be extracted with CCl4. The false increase in the T concentration measured in a direct method appears to be most prominent in the first weeks after birth, although some discrepancy persists through the first 2 months. We studied one direct assay only. Until more information is available about other direct assays, we suggest that assays of plasma T be performed after purification of steroid in plasma of all infants younger than 2 months.

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References