Total Neutral 17-Ketosteroids

Clinical Method for Measurement

Marian S. Kafka and Philip K. Bondy

With increasing demand for the determination of urinary total neutral 17-ketosteroids as a diagnostic tool, a need has developed for a simple, rapid method suitable for clinical use. Such a method was published by Drekter et al. (1) and modified by Masuda and Thuline (2). We have further modified the latter method to permit a larger number of samples to be run at one time. We have substituted equipment which is nonbreakable and less cumbersome, and reduced the number and delicacy of manual operations by making fewer transfers and by simplifying certain steps in the procedure.

MATERIALS AND EQUIPMENT

All chemical reagents are of analytical reagent grade.

1. Polyethylene screw-cap bottles of 200-ml. capacity.
2. Mechanical shaker.
3. Coleman Junior Spectrophotometer with modified adapter.
4. Concentrated HCl.
5. Constant temperature water bath.
7. NaOH pellets.
8. Na₂SO₄, anhydrous.
9. Pyrex culture tubes, without lip, 15 x 85 mm.
10. Ethyl alcohol.

From the Department of Internal Medicine, Yale University, New Haven, Conn. Supported by Grant A-254 of the National Institute of Arthritis and Metabolism of the National Institutes of Health, and an institutional grant from the American Cancer Society. We are grateful to Mrs. Laura Gagnon for her technical assistance. The dehydroepiandrosterone was donated by the Schering Corporation. Received for publication October 20, 1956.
12. Dehydroepiandrosterone. A standard solution containing 10 μg. per 0.2 ml., prepared in absolute ethyl alcohol.
13. Amyl acetate.

The Pyrex culture tubes are selected to fit the adapter of the Coleman Junior Spectrophotometer and calibrated with a dilute solution of methyl red to agree within ±0.02 optical density units.

Meta-dinitrobenzene is purified by dissolving 30 Gm. of the commercial reagent in a slight excess of ethyl alcohol by warming. When the m-dinitrobenzene is in solution, a pinch of Norite is added and the mixture filtered through a warm Buchner funnel containing filter paper covered with a layer of fine-milled asbestos. The filtrate is cooled until the m-dinitrobenzene has crystallized out. The crystals are dried, weighed, then further purified by the method of Callow et al. (3).

**METHOD**

Pipet 10 ml. of urine into a polyethylene bottle fitted with a screw-cap. Add 3 ml. of concentrated HCl and mix. With the cap screwed on lightly, incubate for 12 minutes at 85° in a water bath. Cool rapidly. Add 40 ml. of ethyl ether to the bottle, cap tightly, and shake for 10 minutes on a horizontal shaker (e.g., Eberbach, Ann Arbor, Mich.) in such a way that the aqueous and ether layers mix well but splashing is minimized. Aspirate off the urine (lower) layer. Add 20 ml. of 2.5N NaOH and shake for 5 minutes. Aspirate off the layer of NaOH. Add 20 ml. of distilled water and shake for 5 minutes. Aspirate off the aqueous layer, adding, when necessary, a little anhydrous sodium sulfate to remove traces of water. Detection of the interphases is facilitated if a strong lamp is placed behind the plastic bottle during aspiration. Pipet five 5-ml. aliquots of ether extract into the calibrated culture tubes. Three aliquots constitute triplicate samples of the unknown; two aliquots serve as pigment blanks. Pipet 0.2 ml. of dehydroepiandrosterone standard solution into each of two calibrated tubes. Evaporate the ether aliquots and standard tubes to dryness.

Prepare Solution A immediately before using by mixing two parts of 95% ethyl alcohol, three parts of 5.0 N NaOH (aqueous), and two parts of 2% alcoholic solution of m-dinitrobenzene. Solution B can be prepared once daily by mixing four parts of 95% ethyl alcohol with three parts of 5.0 N NaOH. To each of the dried urine pigment blanks add 0.7 ml. of Solution B and shake. To the dried samples and standards add 0.7 ml. of Solution A and shake well. Similarly, the reagent blank is prepared by adding 0.7 ml. of Solution A to an empty calibrated tube.
Incubate all the tubes in the dark at room temperature for 90 minutes. Just before this period ends, prepare Solution C by mixing one part of amyl acetate with one part of 75% ethyl alcohol. When the 90 minutes are over, add 2 ml. of Solution C to all tubes, stopper with foil-covered corks and shake vigorously. Put the test tubes in the dark for 8 minutes.

Fig. 1. Diagram of cuvet holder for Coleman Junior Spectrophotometer. The figure shows the front half of the holder cut away. The adapter can easily be made from 1" birch dowel. Measurements are in centimeters.
and read at wavelength of 510 m\(\mu\) on the Coleman Junior Spectrophotometer. The Coleman adapter must be modified so that the light beam passes through only the top (solvent) layer in the test tube. This can be accomplished by making a wooden adapter of the specifications given in Fig. 1.

**CALCULATIONS**

Absorbance of sample – absorbance of pigment blank

\[
\text{Absorbance of standard} = \frac{0.01 \text{ mg. of KS (in standard)}}{V_n \times 10 \text{ ml.}}
\]

\[\times \text{ total urine volume in 24 hours} = \text{mg. neutral 17-ketosteroid in 24 hr. urine specimen. The dilution correction factor} = 0.008; \text{ consequently, the equation can be simplified as follows:}
\]

\[
\frac{\text{OD}_u - \text{OD}_b}{\text{OD}_n} \times 0.008 \times V_u = \text{mg./24 hr.}
\]

**EXPERIMENTS AND RESULTS**

The duration of hydrolysis in our method is greater than that used by Masuda and Thuline, because the liquid inside the plastic bottles heats less rapidly than the same liquid contained in glass vessels.

When aliquots of four 24-hour urine specimens were run simultaneously in glass vessels and polyethylene bottles, the values obtained were in good agreement. For each specimen duplicate aliquots were run in glass and polyethylene. The results are given in Table 1.

The validity of premixing the Zimmermann reagents as described above was determined by comparing the intensity of color formed in duplicate aliquots of eight 24-hour urine specimens after adding the reagents one by one with that observed with the combined Solutions A and B. Table 2

**Table 1. Comparison of Color Developed in Urines Hydrolyzed in Various Types of Vessels**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total 24-hr. vol. (ml.)</th>
<th>17-KS in 24-hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glass (mg.)</td>
</tr>
<tr>
<td>R</td>
<td>1300</td>
<td>8.3</td>
</tr>
<tr>
<td>K</td>
<td>850</td>
<td>11.5</td>
</tr>
<tr>
<td>I</td>
<td>445</td>
<td>8.9</td>
</tr>
<tr>
<td>C</td>
<td>615</td>
<td>5.0</td>
</tr>
</tbody>
</table>
shows the total number of milligrams of 17-ketosteroid obtained by the two types of treatment. The variation in the values obtained by the two different methods of adding Zimmermann reagents lies within the variation in the reproducibility found in our laboratory when running the same urine on different days. The temperature at which the Zimmermann color develops may vary between 25° and 35° without altering the intensity of

Table 2. Effect of Combining Zimmermann Reagents on the Intensity of Color Developed

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total 24-hr. vol. (ml.)</th>
<th>17-KS in 24 hr.</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Separate reagents (mg.)</td>
<td>Combined reagents (mg.)</td>
</tr>
<tr>
<td>M</td>
<td>1350</td>
<td>16.9</td>
<td>17.7</td>
</tr>
<tr>
<td>C</td>
<td>1930</td>
<td>20.9</td>
<td>21.5</td>
</tr>
<tr>
<td>H</td>
<td>1210</td>
<td>15.5</td>
<td>15.0</td>
</tr>
<tr>
<td>B</td>
<td>1950</td>
<td>11.0</td>
<td>10.5</td>
</tr>
<tr>
<td>W</td>
<td>1425</td>
<td>11.9</td>
<td>11.4</td>
</tr>
<tr>
<td>O</td>
<td>890</td>
<td>9.5</td>
<td>8.9</td>
</tr>
<tr>
<td>S</td>
<td>342</td>
<td>2.7</td>
<td>2.9</td>
</tr>
<tr>
<td>V</td>
<td>555</td>
<td>10.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Mean ± s.e.:</td>
<td></td>
<td>-0.1 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Lack of influence of the temperature at which color development takes place. Note also close adherence to Beer’s law.
the final color developed, at steroid concentrations between 5 and 50 \( \mu g \).

Masuda and Thuline suggested that the standard steroid be added to portions of each urine extract as an “internal standard,” but this proved clumsy and introduced a considerable potential error, since the absorbance of the standard was calculated from the difference between the readings. It proved simpler to analyze a pure steroid as a standard, and the results obtained were comparable to those obtained by the original method.

Sodium hydroxide was substituted for potassium hydroxide in the Zimmermann reaction because the color developed is a little more intense per unit steroid concentration, making for readings in a more desirable part of the spectrophotometer range in urines with low steroid concentrations.

The absorption spectrum of the Zimmermann color of urinary extracts is similar to that of pure dehydroepiandrosterone (Fig. 3).

---

**Fig. 3.** Absorption spectra of urinary extract (upper curve) and of crystalline dehydroepiandrosterone (lower curve). The absorbance was measured in a Coleman Junior Spectrophotometer using 15 mm. cuvets.
SUMMARY

The Masuda and Thuline method for measuring urinary neutral 17-ketosteroids has been modified by substituting polyethylene bottles and a mechanical shaker for separatory funnels and manual shaking, and by developing the color and reading it in the same test tube. Combining Zimmermann reagents before addition eliminates the necessity for frequently pipeting small volumes. The new method permits the analysis of 10 to 15 urines in a single operation without sacrificing accuracy or specificity.

REFERENCES