THE ESTIMATION OF SMALL AMOUNTS OF CORTICOSTERONE IN RAT PLASMA

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(Received for publication, November 14, 1957)

Many of our investigations involving adrenal physiology have been carried out in the rat (1–4). Since corticosterone is the preponderant adrenal steroid found in rat plasma, it became essential to develop a rapid, valid method for its determination.

The Silber-Porter method (5) is inadequate for studies in the rat because it is neither sensitive nor specific enough. The method of Sweat (6), although demonstrating the usefulness of sulfuric acid-induced fluorescence for analysis of plasma corticosteroids, has not been found practical.

Our method uses the double extraction technique of Silber and Porter and a modification of the fluorescence technique described by Sweat (7).

EXPERIMENTAL

Reagents—
Chloroform, Mallinckrodt, A. R.
Sodium hydroxide, 0.1 N.
Ethyl alcohol, redistilled from m-phenylenediamine as recommended by Callow et al. (8).
Sulfuric acid, c.p. grade.
Fluorescence reagent. 2.4 volumes of sulfuric acid are added to 1.0 volume of 50 per cent (v/v) aqueous ethyl alcohol. This reagent is stable.
Corticosterone standard. 25.0 mg. of corticosterone are dissolved in 50.0 ml. of ethyl alcohol. A 1:50 dilution of this standard with water (10.0 γ per ml.) has an absorbance of 0.483 at 247 mp in a 1 cm. cell. This standard is further diluted to give a working standard containing 0.15 γ per ml.

Materials and Methods

Glassware washed with detergent must be rinsed with alcohol and dried before use. An automatic burette may be used to measure the chloroform.
and an autopipette to measure the fluorescence reagent. The fluorescence readings are made in the Coleman model No. 12B or in the Farrand model No. 12A fluorometer equipped with the Corning No. 5113-3389 glass filters as a primary filter and a 540 m\(\mu\) interference filter as a secondary filter. Coleman cuvettes are raised to permit the use of 3 ml.

**Extraction**—Aliquots of 0.3 to 3.0 ml. of peripheral or 0.02 to 0.1 ml. of adrenal venous plasma are measured into a 50 ml. glass-stoppered conical centrifuge tube and the volume is made up to 3 ml. with distilled water. A blank, consisting of 3 ml. of water, and standards, containing the desired volume of working standard (0.5 to 3.0 ml.) in a total aqueous phase of 3 ml., are carried through the procedure. A 45.0 ml. portion of chloroform is introduced into each tube and the tubes are shaken for 15 seconds. The tubes are then centrifuged for 5 minutes at 2500 r.p.m., and each aqueous layer is removed by aspiration as described by Silber and Porter (5).

**Washing**—Aliquots of 4.5 ml. of 0.1 \(\text{n}\) NaOH are added and the tubes are shaken for 15 seconds and centrifuged. Each aqueous layer is removed as above.

**Extraction by Reagent**—Two 15.0 ml. aliquots of chloroform extract are measured into clean 50 ml. glass-stoppered centrifuge tubes and 3.0 ml. of fluorescence reagent are added to each tube. The tubes are shaken vigorously for 15 seconds, centrifuged at 2500 r.p.m. for 5 minutes, and the chloroform is removed as completely and as carefully as possible without removing the fluorescence reagent.

**Fluorescence**—The sulfuric acid layer is pipetted into a fluorometer cuvette which is allowed to stand at room temperature. 2 hours after the extraction by reagent the fluorescence is measured in the fluorometer.

**Calculations**—The standard curve is plotted on linear graph paper and the amount, \(A\), in the sample is read from that graph. The concentration, \(C\), in micrograms per 100 ml., is obtained by the formula: \(C = A \times 100/V\), where \(V\) is the number of ml. of plasma used.

**Results**

**Specificity**—The use of a simple technique for the determination of rat plasma corticosterone evolved from the study of the effect of the ratio of sulfuric acid to 50 per cent ethyl alcohol on fluorescence (Fig. 1). It is seen from Fig. 1 that, at a ratio of 2.4 volumes of sulfuric acid to 1.0 volume of 50 per cent aqueous alcohol, corticosterone fluoresces about five times as much as hydrocortisone. Furthermore, rat plasma follows the first part of the corticosterone curve closely, which indicates that a certain amount of specificity might be expected at lower ratios. This is supported by a crude fluorescence spectrum obtained by the use of four different interference filters and given in Fig. 2.
Fig. 1. Influence of the ratio of sulfuric acid to 50 per cent ethyl alcohol on the fluorescence of corticosterone, hydrocortisone, and rat plasma.

Fig. 2. Fluorescence spectrum of corticosterone, hydrocortisone, and rat plasma with an acid to alcohol ratio of 2.4.
The specificity of the method was further studied in two ways: (a) by estimating the fluorescence of other steroids, and (b) by physiological experiments with rats.

**TABLE I**

*Relative Fluorescence of Steroids*

The steroids below were carried through the procedure; their fluorescence is expressed as per cent corticosterone.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Fluorescence</th>
<th>Steroid</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>100.0</td>
<td>17-Hydroxypregnenolone</td>
<td>0.0</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>19.8</td>
<td>Pregnandiol</td>
<td>1.0</td>
</tr>
<tr>
<td>Tetrahydrocortisone</td>
<td>2.0</td>
<td>Androsterone</td>
<td>0.0</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.1</td>
<td>Dehydroepiandrosterone</td>
<td>0.0</td>
</tr>
<tr>
<td>11-Dehydrocorticosterone</td>
<td>0.1</td>
<td>4-Androstene-3,17-dione</td>
<td>0.0</td>
</tr>
<tr>
<td>11-Deoxy corticosterone</td>
<td>0.2</td>
<td>Estrone</td>
<td>3.8</td>
</tr>
<tr>
<td>17-Hydroxy-11-deoxycorticosterone</td>
<td>0.1</td>
<td>Estradiol</td>
<td>14.2</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.0</td>
<td>Estriol</td>
<td>0.0</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone</td>
<td>0.0</td>
<td>Hexestrol</td>
<td>0.0</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.6</td>
<td>Diethylstilbesterol</td>
<td>0.0</td>
</tr>
</tbody>
</table>

![Fig. 3. Effect of bilateral adrenalectomy on the corticosterone level of rats. Each point represents the mean of five animals; the standard deviations are given in brackets.](http://www.jbc.org/)

The result of the chemical experiment (Table I) shows that, of all the steroids investigated, only hydrocortisone and estradiol fluoresce appreciably.
The lack of plasma fluorescence after adrenalectomy (Fig. 3) indicates that most of the fluorescence obtained in the plasma of the intact rat is derived from adrenal steroids. From Sweat's data\textsuperscript{1} the fluorescence contribution of hydrocortisone in rat plasma can be estimated to be of the order of 6 per cent at an acid to ethanol ratio of 2.4; our investigations indicate a contribution of approximately 5 per cent at that ratio.

The fluorescence due to endogenous estrogen is negligible because the values of plasma corticosterone for oophorectomized-adrenalectomized rats are of the same order as those for adrenalectomized rats (Table II).

**Characteristics of Fluorescence**—The fluorescence spectrum obtained with

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**Table II**

Influence of Oophorectomy on Fluorescence of Plasma in Adrenalectomized Rats

Bilateral oophorectomy was performed 6 weeks before the final bleeding. The time between bilateral adrenalectomy and the final bleeding is given in days.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Ovaries</th>
<th>Days after adrenalectomy</th>
<th>Corticosterone γ per 100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>887</td>
<td>Intact</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>888</td>
<td>&quot;</td>
<td>7</td>
<td>2.4</td>
</tr>
<tr>
<td>883</td>
<td>&quot;</td>
<td>14</td>
<td>2.4</td>
</tr>
<tr>
<td>884</td>
<td>&quot;</td>
<td>14</td>
<td>3.1</td>
</tr>
<tr>
<td>885</td>
<td>Removed</td>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td>886</td>
<td>&quot;</td>
<td>7</td>
<td>3.2</td>
</tr>
<tr>
<td>881</td>
<td>&quot;</td>
<td>14</td>
<td>2.2</td>
</tr>
<tr>
<td>882</td>
<td>&quot;</td>
<td>14</td>
<td>1.8</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Development of fluorescence in function of time.
the filter system described in the method is given in Fig. 2. It is similar to spectra obtained by other workers (7, 9) under similar conditions. From Fig. 4 it is seen that the development of fluorescence is virtually completed 2 hours after the extraction of the chloroform aliquot with the fluorescence reagent.

Corticosterone concentrations ranging from 0.05 to 5.0 μg plotted against fluorescence produce a linear relationship (Fig. 5). This indicates the absence of self-absorption in that range.

The absence of quenching substances in rat plasma is suggested by the linear relationship obtained when fluorescence is plotted against plasma volumes ranging from 0.2 to 3.0 ml. (Fig. 5).

![Fig. 5. Relationship between fluorescence and plasma volume, and fluorescence and corticosterone concentration.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Plasma, ml</th>
<th>Corticosterone</th>
<th>Mean per 100 ml</th>
<th>Corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
<td>Recovered</td>
</tr>
<tr>
<td>1.0</td>
<td>0.333 ± 0.012</td>
<td>0.250</td>
<td>0.555 ± 0.002</td>
</tr>
<tr>
<td>0.2</td>
<td>0.069 ± 0.002</td>
<td>0.200</td>
<td>0.251 ± 0.005</td>
</tr>
</tbody>
</table>

TABLE III

*Analysis of Corticosterone in Small Quantities of Peripheral Plasma*

All data are expressed in micrograms.
Sensitivity and Reliability—Data on the sensitivity of the method are reported in Table III; the results obtained with 1.0 ml. of plasma were 33.3 ± 1.2 γ per 100 ml. of corticosterone with a recovery of 88.8 per cent. These do not differ significantly from those obtained with 0.2 ml. of plasma, i.e. 34.5 ± 1.0 γ per 100 ml. with a 91.0 per cent recovery. Quantities as small as 0.2 ml. are clearly sufficient for analysis under these conditions.

In order to test the reliability of the method, samples of a rat plasma pool (Long-Evans male rats) were placed in small test tubes and stored in the frozen state. Determinations made on 8 different days showed a mean of 29.9 γ of corticosterone per 100 ml. with a standard deviation of 1.15 and 95 per cent confidence limits of 2.6 γ per 100 ml.

SUMMARY

A method for the fluorometric determination of rat plasma corticosterone has been described. The method is sensitive, for as little as 0.2 ml. of plasma permits a duplicate determination. Data are presented which indicate the specificity and reliability of the method.

Some of the steroids used in this study were generously donated by K. Pfister, Merck and Company, Inc., Rahway, New Jersey. Grateful acknowledgment is made to Sarah Cheiker for technical assistance.

BIBLIOGRAPHY

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