ESTIMATION OF 17,21-DIHYDROXY-20-KETO-STEROIDS IN PLASMA BY A MICRO-MODIFICATION OF THE SILBER-PORTER ASSAY

By

A. M. Riondel, M. G. Sanz and A. F. Muller

In 1950 Porter & Silber (1950) described a colour reaction between 17,21-dihydroxy-20-keto-steroids and phenylhydrazine. Four years later, the same authors (Silber & Porter, 1954) developed a relatively simple method for the routine clinical estimation of these corticosteroids in human plasma. The procedure involves four steps: (1) extraction with 5 volumes of chloroform, (2) washing with dilute alkali, (3) extraction of an aliquot of the alkali-washed chloroform extract with alcoholic phenylhydrazine-sulfuric acid reagent, and (4) measurement of optical density in micro-cuvettes in the Beckman spectrophotometer after heating the sample to 60° for 30 minutes.

We have shown in a preliminary communication (Muller et al., 1955) a very good agreement between the relatively simple method of Silber & Porter and the more elaborate procedure of Nelson & Samuels (1952), requiring adsorption of the plasma extract on a Florisil column with subsequent elution. Since both techniques employ the phenylhydrazine-sulfuric acid reagent for identification and estimation of 17,21-dihydroxy-20-keto-steroids, they are subject to the same limitations of specificity as are inherent in the Porter-Silber reaction itself, but the plasma extract contains fewer chromogens in the Nelson-Samuels assay.

Wallace et al. (1955) have recently presented their first result in the clinical application of this simplified Silber-Porter method.

The present report is concerned with the description of a micro-modification, using only 2 ml. of plasma, of the Silber-Porter method. A comparison of this

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new technique with the well established Nelson-Samuels procedure is also presented.

METHOD AND RESULTS

Reagents and Equipment
1. Chloroform (Merck reagent).
2. 0.1 N sodium hydroxyde.
3. Diluted sulfuric acid; 310 ml. of concentrated sulfuric acid (Merck reagent) diluted with 190 ml. of water.
4. Blank reagent; 100 ml. of diluted sulfuric acid mixed with 50 ml. of absolute ethanol.
5. Phenylhydrazine-sulfuric acid reagent with ethanol; 65 mg. of phenylhydrazine hydrochloride recrystallized from ethanol, dissolved in 150 ml. of blank reagent.
6. Aqueous cortisol standard, 20 µg./ml.
7. Centrifuge tubes with ground glass stoppers. 20 ml. capacity, 15 cm. by 15 mm.
8. Centrifuge tubes with ground glass stoppers, 14 ml. capacity, 13 cm. by 14 mm.
9. Dumb-bell shaped tubes with ground glass stoppers (Fig. 1).

Fig. 1.
Dump-bell shaped tube used for the extraction into the colour reagent. (Dimensions in mm.)
10. Test tubes, 1.5 ml. capacity, 8 mm. diameter.

11. Micro cuvettes for Beckman Model DU spectrophotometer, Corex cells, light path 10 mm., capacity 0.5 ml.

**Technique**

5 ml. of blood are gently shaken with 0.2 ml. of 0.2% heparin, immediately centrifuged and the plasma separated from red cells.

**Extraction.** To each 2 ml. sample of plasma in tube No. 7, 1 µg. of cortisol is added (Sanz micropipette*). The sample is vigorously shaken with 10 ml. of chloroform for 20 seconds in a shaking-machine, and then centrifuged.

**Washing.** The centrifuged chloroform extract is transferred by means of a syphon to tube No. 8 and then washed with 0.4 ml. of 0.1 N sodium hydroxide by shaking in a shaking-machine for 15 seconds. After brief centrifugation, the alkaline wash is removed by aspiration with a capillary tube and discarded.

**Extraction by Reagent.** The washed chloroform extract is divided into two 4 ml. aliquots, each of which is placed in a dumb-bell shaped tube (No. 9). To one is added 0.5 ml. of phenylhydrazine-sulfuric acid reagent with ethanol (No. 5), to the other 0.5 ml. of blank reagent (No. 4). The tubes are vigorously shaken in a shaking-machine for 2 minutes and then centrifuged. The supernatant reagent layer, located in the middle section of the dumb-bell tube, is carefully transferred by aspiration with a capillary tube to a 1.5 ml. test tube (No. 10).

**Colour Reaction.** After heating in a water bath at 60° for 30 minutes and cooling, the solutions are transferred to micro-cuvettes, and the optical densities read at 370, 410 and 450 mµ: the unknown phenylhydrazine-sulfuric acid reagent with ethanol sample against the corresponding sample containing only the reagent blank.

**Standards.** Cortisol in aqueous solution, 0.5, 1.0, 1.5 and 2.0 µg. are added to 4 samples of 2 ml. plasma, which previous to the addition of the steroid have been washed with chloroform. These plasma standards are re-extracted with chloroform and then carried through the same procedure as the unknown plasma samples, thus serving as recovery standards.

**Blanks.** Two 4 ml. aliquots of pure chloroform are treated in exactly the same manner as the 4 ml. aliquots of the plasma chloroform extracts.

**Calculation.** Instead of a single reading at 410 mµ as described by Silber & Porter (1954), the correction equation introduced by Allen (1950) is used, as recommended by Hertoghe et al. (1955). In view of the very low optical density of the blanks, the results, calculated with the correction equation of Allen, are not significantly influenced by these acid-blanks.

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* Demonstrated at the International Congress of Clinical Chemistry, September 1956, New York.
Specificity

Concerning the specificity of this micro-method, reference is made to the discussion in the paper by Silber & Porter (1954). It holds pari passu for our assay.

Precision of Procedure

In agreement with Silber & Porter (1954), we find the estimation less accurate at low plasma concentrations. Hence, we recommend that 1 µg. of cortisol should always be added to the plasma. One of the reasons for this low accuracy is the relatively weak colour obtained with 1 µg. of the recovery standard. Fig. 2 demonstrates clearly, that the optical density per µg., i.e. the slope of the calibration curve, varies according to the step at which the standard is added. With the phenylhydrazine reagent one µg. of pure cortisol develops a colour intensity which is three times stronger than that given by 1 µg. of the recovery plasma standard. This is due to the considerable loss of steroid

![Calibration curves of cortisol standard.](image)

- Curve I, pure cortisol and phenylhydrazine reagent;
- Curve II, pure cortisol in chloroform extracted with the reagent;
- Curve III, pure cortisol added to extracted plasma and carried through the whole procedure.
which occurs in each extraction step. Curve III being flat, important quantitative differences may occur with relatively small variations in the optical density readings. It is therefore of extreme importance to have an excellent spectrophotometer, since the precision of the measurements depends largely upon the optical performance of the instrument. Nevertheless, from the technical point of view, this remains a great disadvantage of the Silber-Porter method.

Table 1 shows the standard deviations of 41 duplicate estimations, done by the same technician. Since the relative precision is less for low values, the plasma samples have been divided arbitrarily into 3 groups, according to the levels found. The arithmetical mean and the standard deviation within duplicates were calculated separately for each group, as well as for all the values together. The over-all standard deviation is ±2.2 µg./100 ml. Engel et al. (1955) obtained a similar precision when using the Nelson-Samuels procedure.

**Table 1.**

<table>
<thead>
<tr>
<th>Range of plasma levels µg./100 ml.</th>
<th>Number of samples</th>
<th>Arithmetic mean µg./100 ml.</th>
<th>Standard deviation of duplicates µg./100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–10</td>
<td>16</td>
<td>5.1</td>
<td>±1.5</td>
</tr>
<tr>
<td>10–30</td>
<td>18</td>
<td>20.1</td>
<td>±2.4</td>
</tr>
<tr>
<td>&gt; 30</td>
<td>7</td>
<td>43.5</td>
<td>±2.8</td>
</tr>
<tr>
<td>All samples</td>
<td>41</td>
<td>18.3</td>
<td>±2.2</td>
</tr>
</tbody>
</table>

In a series of 12 duplicate estimations, done on separate occasions and by two different technicians, the standard deviation within duplicates was only slightly greater (±2.8 µg./100 ml.).

**Comparison between the Micro-Modification of the Silber-Porter Assay and the Nelson-Samuels Technique (Statistical Analysis)**

One hundred and four plasma samples were analyzed by both methods. The results have been grouped according to the level found with the micro-modification of the Silber-Porter method: 0–10, 10–30, or > 30 µg./100 ml. (Fig. 3).

(1) A t-test indicates that the mean of the differences between corresponding Silber-Porter and Nelson-Samuels values probably does not differ from zero (P > 0.05).

(2) If the Nelson-Samuels and the Silber-Porter values are regarded as duplicate determinations, their standard deviations are, for the three range
Corticosteroid levels in 104 plasma samples analyzed by the method of Nelson & Samuels (1952) and by the modified Porter-Silber technique described in this paper. The heavy lines represent the regression lines and the interrupted lines correspond to the fiducial limits. The thin line with 45° slope represents the relation $Y = x$.

Groups, similar to those shown in Table 1 for the »true« duplicates, namely, $\pm 2.9$, $\pm 2.1$, and $\pm 1.2 \mu g./100$ ml., respectively.

(3) The coefficient of determination, or square of the correlation coefficient (Linder, 1951), is found to be 0.34 in the low range of plasma levels, 0.66 in the middle range, and 0.87 in the upper range group. All three correlations are highly significant ($P < 0.001$).

(4) The regression lines shown in the scatter diagram (Fig. 3) have been calculated under the assumption that the Nelson-Samuels results ($x$) are the accurate »true« values, and the Silber-Porter results ($y$) the dependent variate. Fiducial limits, also shown in the graph, have been calculated, for $P = 0.05$, as $Y \pm ts$, where $Y$ is the value expected from the regression equation $Y = a + bx$, and $s$ the standard deviation corresponding to the residual variance $s^2 = (1-B) \cdot S_{yy}/(N-2)$. In the latter formula, $B = \text{coefficient of determination (Linder, 1951)}, S_{yy} = \text{total sum of squared deviations of the } y \text{'s}, \text{ and } N = \text{number of samples}.$
The regression analysis confirms the preceding statistical calculations. However, it should be noted that the expected Silber-Porter values below 10 µg./100 ml. amount only to 58% of the Nelson-Samuels values, whereas in the two higher range groups the regression lines do not differ significantly from the »theory«, \( Y = x \). The residual standard deviation in the two lower range groups are ± 2.8 and ± 2.9, respectively. These values are very similar to the standard deviation of »true« duplicates (Table 1). In other words, the Silber-Porter values do not differ from the values predicted by the regression lines any more than do duplicate determinations among themselves.

**DISCUSSION**

On the average, the new micro-modification has given in our hands as precise and accurate results as the original macro-method proposed by Silber & Porter (1954) (Muller et al., 1955). The standard deviations of duplicate determinations are satisfactory and compare favourably with those of the Nelson-Samuels technique. However, when expressed as a percentage of the result, the standard deviation for values below 10 µg./100 ml. becomes rather large. But this criticism, which is inherent in the method, results from the considerable loss of steroid occurring during the various extraction steps, and it also applies to the original procedure. This necessitates the use of recovery plasma standards, which from the technical point of view is never very desirable.

The comparison of this micro-method with the well established but meticulous Nelson-Samuels technique is also very satisfactory. So far our clinical results have been encouraging, like those of others (Wallace et al., 1955). The various statistical procedures used have established a close correlation between the results obtained by the two methods. Yet, one objection must be raised: the agreement between the two assays is not perfect in the low range of values (below 10 µg./100 ml.). The Silber-Porter measurements are usually lower (58% of the Nelson-Samuels values). However, considering its simplicity and rapidity, this method can be recommended.

**SUMMARY**

A micro-modification of the Silber-Porter technique for the estimation of corticosteroids in blood has been described. This new method presents all the advantages of the original technique, being rapid, simple and accurate, and yet requires only 2 ml. of plasma for a single determination. Moreover the values agree very well with those obtained by the more tedious and time-consuming procedure of Nelson & Samuels. So far our clinical experience with this method has been excellent.
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REFERENCES