
Quantitative Paper Chromatography of Reducing Steroids of the Adrenal Cortex

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The methods commonly employed for the determination of steroids furnish only limited information when applied to adrenal extracts. Not only is it impossible to estimate individual members of the group of biologically active corticoids in a mixture, but the presence of many closely related, but inactive, compounds in gland extracts renders the overall determination of reducing or formaldehydegenic activity largely valueless. Since paper chromatography affords a ready means of resolving the complex mixture of cortical steroids into single compounds or groups of closely related compounds, the quantitative evaluation of particular members of the cortical group thus becomes feasible.

It was desired to develop a method which would enable the steroids to be located on the chromatogram and to be determined colorimetrically by elution of the product of the reaction, thus avoiding the necessity of running reference strips or relying on the $R_f$ values for the elution of the appropriate portions. A survey of the known reactions of steroids applicable to paper chromatograms indicated that they either lacked sensitivity or that they were not quantitative. The arsenomolybdate reaction reported previously (Schwarz, 1952a) was found to be satisfactory in both respects. The technique described below is simple and does not require any elaborate equipment. It readily lends itself to routine analysis of mixtures of cortical steroids and it should prove useful for the determination of reducing cortical steroids in blood and urine.

EXPERIMENTAL

Chromatography

The solvent systems described by Zaffaroni, Burton & Keuttman (1950) and by Bush (1952) have been used. With the former the more polar and the less polar steroids have to be resolved on separate strips, one being run in toluene/propane glycol, the other in benzene/formamide. The time required for satisfactory separation depends on a number of factors, but it is reasonably constant under strictly standardized conditions. In the experience of the author, 6–7 hr. development with benzene of the formamide-impregnated strips gives a separation of the fast-running steroids satisfactory for quantitative purposes, while a running time of about 100 hr. in toluene ensures a reasonable separation of hydrocortisone (17-hydroxy cortisol) from very polar material at the top of the chromatogram.

Several of the solvent systems described by Bush (1952) enable all the biologically active steroids to be separated from each other on one strip and in a much shorter time. The practical difficulties associated with chromatographing at 34° have been overcome by the use of the "chromatocoil" (Schwarz, 1952b), in which the paper strip is accommodated in spiral form in a container which can readily be placed in an incubator. A paper strip 50 cm. in length, or longer, can easily be fitted into an apparatus measuring no more than 5 cm. diameter x 10 cm. long. The use of the ascending technique has the advantage that development stops automatically when the solvent front reaches the end of the strip, thus making accurate timing unnecessary. Moreover, with the same length of strip, the steroids will always travel approximately the same distance. While chromatographic development is somewhat slower than in the descending arrangement, saturation is effected much more quickly owing to the dimensions of the apparatus. Thus a complete run can be carried out in 5–6 hr.
The biologically active steroids separated chromatographically in any of the solvent systems referred to are still associated on the chromatogram with biologically inactive steroids. Further resolution can be achieved by rechromatographing each group of steroids separately and for longer periods (Zaffaroni & Burton, 1961).

After chromatography the paper strips are air-dried, or, in the case of propylene glycol or formamide-saturated strips, they are placed in a stream of warm air (about 70°) for 15 min. The dry strips are then ready for treatment with arsenomolybdate reagent.

**Reagent.** This is prepared according to Nelson (1944). A solution of 25 g ammonium molybdate in 450 ml water is mixed with 21 ml conc. H$_2$SO$_4$, and a solution of 3 g Na$_2$HAsO$_4$·7H$_2$O in 25 ml water is added. The mixture is incubated at 37° for 48 hr. and filtered. It is stored in a glass-stoppered brown bottle.

**Application of reagent and incubation.**

Many different methods of impregnating the paper strips with the reagent were tried, but all of them were found wanting in one or more respects, e.g. uneven wetting of different parts of the strip; adsorption of arsenomolybdate on one edge; loss of steroids; or longitudinal displacement of the steroids. (Spraying was ruled out on account of the nature of the reagent.)

**Qualitative method.** The following method was found to give the most satisfactory results for qualitative work, in spite of the loss of a small amount of steroid matter.

A length of thin, plain-weave, bleached cotton fabric (25 threads/cm, 0-72 g./100 sq.cm) is boiled in distilled water for about 1 hr., rinsed and dried. A strip, about 1-2 cm wide, is cut out and placed on a length of flexible plastic material (a piece of washed X-ray film was found suitable) wrapped round a glass plate. Sufficient reagent is then run along the length of the fabric from a test pipette to form a continuous film. The chromatogram strip is placed on another glass plate, and the wet fabric supported by the X-ray film and glass plate is inverted over it quickly and evenly, without exerting undue pressure. After removing the top glass plate, the X-ray film can be bent backwards, thus enabling the fabric to be separated from the paper strip. By this means the test strip is moistened evenly and simultaneously along the whole length. A ring of Whatman no. 3 filter paper, having a short ‘tail’ at either end, is then placed around the test strip. The paper ring is kept damp by allowing the tails to dip into water during the incubation. After placing a warm glass plate over the test strip and paper ring, the whole arrangement is incubated on a heavy metal base in an electric oven at 70° for 30–60 min. About 1–2 μg of deoxytocorticosterone (DOC)/sq.cm. can be detected by this method, which is thus considerably more sensitive than other methods commonly employed.

**Quantitative method.** For quantitative purposes the above method has to be modified slightly, since a small amount of steroid matter diffuses on to the fabric and is thus lost. The procedure adopted is as follows.

The chromatogram strip is covered by another paper strip of the same length and width and the two strips are placed on a glass plate. Two layers of fabric are used in order to increase the volume of reagent. Flooding of the fabric is to be avoided. After inverting the damp fabric over the double paper strips, a film of reagent should be formed between the test strip and the supporting glass plate, and between the two paper strips. The plate supporting the X-ray film is carefully removed, leaving the latter in position. The fabric can then be separated easily from the paper strips as shown in Fig. 1. After surrounding the strips by a paper ring and covering them with a glass plate (previously warmed to 76–77°) the double plates are placed on the metal base heated to the same temperature, and incubated at 70° for 60 min. It is important to keep the surrounding paper ring damp during the incubation, as concentration of the reagent results in high blank readings.

![Fig. 1. Diagrammatic representation of arrangement for application of reagent: a, glass plate; b, test strip and cover strip; c, double layer of fabric; d, X-ray film. The glass plate supporting the X-ray film has been removed to permit bending of the X-ray film for easier separation of fabric from paper strip.](image)

**Incubation temperature.** The temperature of incubation is chosen so that the rate of reaction is sufficiently slow after 1 hr. to permit reproducible results to be obtained. Longer incubation periods have been found unsuitable on account of the greater evaporation from the strip, while shorter periods at a higher temperature increase the uncertainty associated with the raising of the temperature of the cold glass plate and strips to the desired level. Fig. 2 shows the rate of reaction at three different temperatures. It is seen that the reaction is almost complete after 1 hr. at 70°, and this therefore represents the optimum temperature which ensures both accuracy and sensitivity.

**Colorimetry.**

At the end of the incubation the cover plate is removed, the damp strips are laid side by side and appropriate lengths are severed (contact with metallic objects must be avoided) for elution. Lengths of 2-5–5 cm. have usually been found suitable. A similar length is taken from above the starting line to act as a blank. The corresponding lengths are superimposed and eluted together, one end of each pair being
allowed to dip into a shallow Perspex trough, while the other hangs into a colorimeter tube. Alternatively, the top end can be held between two horizontal microscope slides. The molybdenum blue complex is eluted with distilled water and the eluate is made up to a definite volume (4-5 ml.). The optical density is read in a photoelectric colorimeter at 650-660 mμ. against the eluate of the blank strip.

The comparatively high optical absorption of the latter (equivalent to about 2 μg. of DOC/sq.cm.) is not due to the reagent or to impurities in the paper, but results from the partial hydrolysis of the cellulose. The blank is, however, constant over the whole length of the chromatogram strip and is proportional to the length of paper eluted. Thus the appropriate allowance can readily be made for test strips which are longer or shorter than the blank strip.

The blue colour is stable over long periods, but since the reaction is not quite complete after 1 hr. incubation, there is a tendency for the optical absorption to increase slightly on keeping for several hours.

If the amount of reducing material is greater than about 30 μg. of DOC/sq.cm. of strip, a small fraction of the molybdenum blue complex remains adsorbed on the cellulose and cannot be eluted with the volume of water used. In the experience of the author, this is not a serious limitation as such high concentrations are not normally encountered. However, the fraction adsorbed is reasonably constant and allowance can be made for it by running suitable standards.

RESULTS

Solutions of molybdenum blue obey the Beer-Lambert law, but since, in practice, there is a slight deviation from the linear relationship between reducing material present on the strip and molybdenum blue recovered, a standard curve has to be plotted. Moreover, since the reducing power is different for each steroid, separate curves have to be constructed for all corticoids to be estimated, or conversion factors have to be used. The latter procedure will be found sufficiently accurate for most purposes.

Table 1. Relative reducing power of different steroids

(60 min. incubation. DOC=1.0.)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Arsenomolybdate</th>
<th>Phosphomolybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOC</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Compound A</td>
<td>0.87</td>
<td>0.84</td>
</tr>
<tr>
<td>Cortisone</td>
<td>—</td>
<td>0.74</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.69</td>
<td>—</td>
</tr>
<tr>
<td>Metyltestosterone</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.33</td>
<td>0.30</td>
</tr>
<tr>
<td>Pregn-4-ene-20β:21-diol-3:11-dione</td>
<td>0.17</td>
<td>—</td>
</tr>
<tr>
<td>17-Ethylandrosten-4-ene-17α-ol-3-one</td>
<td>—</td>
<td>0.17</td>
</tr>
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</table>

In Table 1 the relative reducing powers of several steroids are compared with the values obtained by Heard & Sobel using the phosphomolybdate method (Heard & Sobel, 1948).

The agreement between the two sets of values suggests that the various functional groups of the steroid molecule make the same fractional contribution to the total reducing power in both methods. It can therefore be assumed that the same general considerations govern the reducing activity of steroids under the milder conditions of the arsenomolybdate technique as have been found by Heard & Sobel to obtain in the phosphomolybdate method.

The slight deviation from the linear relationship between reducing matter present and molybdenum blue formed may be due to local insufficiency or lack of accessibility of reagent, resulting from the limited diffusion possible under the experimental conditions. Accessibility, in particular, may be restricted in the case of hydrophobic steroids like DOC. It is likely to be aggravated by irregular distribution of the steroid which is difficult to avoid when comparatively large volumes of solution are applied to a restricted area by repeated evaporation.

Fig. 3. Reference curve for deoxy cortisol.

Fig. 3 shows the experimental relationship between the amounts of DOC placed on the strip and the optical densities of the eluates. The average of at least three determinations has been used for the plotting of each point. In no case do individual determinations differ by more than 11% from the mean, and the average deviation of forty-six determinations is 3.8%.

As has been shown above, the recoveries fall off as the concentration of DOC/unit area increases. The optical densities have therefore been plotted against concentration/sq.cm. rather than against the total amount of DOC. In using the standard graph it is thus necessary to divide the optical density by the length of the spot (if strips of unit width are used), and to multiply the equivalent amount of DOC by the same value. Since the deviation from a straight
line is small, the spot length need not be determined very accurately.

The method outlined above has been applied to the quantitative analysis of two synthetic mixtures of steroids. Table 2 shows the mean of the determinations, the standard deviation, and the recovery.

Of the many adrenal extracts examined, analytical results obtained with two experimental extracts are given in Table 3.

Table 2. Analysis of synthetic mixtures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Present (µg./ml.)</th>
<th>Found (mean of four determinations) (µg./ml.)</th>
<th>S.D.</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>10</td>
<td>9.75</td>
<td>0.75</td>
<td>98</td>
</tr>
<tr>
<td>E</td>
<td>8.8</td>
<td>8.85</td>
<td>0.58</td>
<td>101</td>
</tr>
<tr>
<td>B</td>
<td>20*</td>
<td>16.25</td>
<td>1.27</td>
<td>---</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>17.20</td>
<td>0.38</td>
<td>86</td>
</tr>
</tbody>
</table>

Mixture II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Present (µg./ml.)</th>
<th>Found (mean of three determinations) (µg./ml.)</th>
<th>S.D.</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>20</td>
<td>19.87</td>
<td>1.18</td>
<td>99</td>
</tr>
<tr>
<td>E</td>
<td>26.4</td>
<td>25.63</td>
<td>0.29</td>
<td>97</td>
</tr>
<tr>
<td>B</td>
<td>13*</td>
<td>11.90</td>
<td>0.33</td>
<td>---</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>37.47</td>
<td>1.14</td>
<td>94</td>
</tr>
<tr>
<td>DOC</td>
<td>20</td>
<td>17.13</td>
<td>0.42</td>
<td>86</td>
</tr>
</tbody>
</table>

* Compound B was not available in crystalline form and was prepared from adrenal extract by paper chromatography. The absolute amount of B present in the synthetic mixtures is therefore not known accurately.

Table 3. Analysis of adrenal extracts

<table>
<thead>
<tr>
<th>Steroids associated with</th>
<th>Extract A (mean of three determinations) (µg./ml.)</th>
<th>S.D.</th>
<th>Extract B (mean of two determinations) (µg./ml.)</th>
<th>S.D.</th>
<th>Rf</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound F</td>
<td>40.2</td>
<td>0.32</td>
<td>33.35</td>
<td>0.78</td>
<td>0.04</td>
<td>1.2</td>
</tr>
<tr>
<td>Compound E</td>
<td>23.8</td>
<td>1.11</td>
<td>17.35</td>
<td>0.64</td>
<td>0.04</td>
<td>1.4</td>
</tr>
<tr>
<td>Unidentified steroid</td>
<td>13.4</td>
<td>0.87</td>
<td>8.2</td>
<td>0.28</td>
<td>0.29</td>
<td>1.6</td>
</tr>
<tr>
<td>Compound B</td>
<td>23.4</td>
<td>1.97</td>
<td>12.45</td>
<td>0.50</td>
<td>0.42</td>
<td>1.9</td>
</tr>
<tr>
<td>Compound A</td>
<td>26.0</td>
<td>1.03</td>
<td>13.1</td>
<td>0.85</td>
<td>0.54</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Extract A is a purified solution of cortical steroids in ethyl acetate; extract B was prepared from A by distilling off the organic solvent in the presence of normal saline and filtering off the precipitate.

The loss of steroid material due to removal of the precipitate would be expected to increase with the diminishing hydrophilic nature of the steroid. This is fully borne out by the experimental results and is shown by the ratio A/B in the last column of Table 3. The increasing Rf values (in toluene-light petroleum/aqueous methanol) are a measure of the decreasing solubility in water.

DISCUSSION

The arsenomolybdate reaction is very sensitive, 1-2 µg. of reducing steroid per sq.cm. being readily detected. In view of the comparatively large blank value of the paper strip itself, it is, however, not possible to determine less than 5 µg. of steroid with any accuracy. The upper limit is set by the local availability of reagent and the adsorption of the blue complex on to the paper. With an average elongation of the steroid spot during chromatography to 2-3 cm., up to 100 µg. can be dealt with satisfactorily. This represents a range of usefulness sufficiently wide for most purposes.

It is apparent from the standard deviations presented in Tables 2 and 3 that agreement between repeated analyses is reasonably good. All individual results obtained are, in fact, within less than 10% of the mean values. In the analysis of the synthetic mixtures the amounts of steroids recovered are, on the whole, satisfactory, although recovery tends to be somewhat lower in the case of the faster-travelling steroids.

The principal weakness of the method lies in the comparatively large optical absorption of the blank strips. Many attempts have been made to increase the accuracy by reducing the blank value, but all have been unsuccessful. Thus, no benefit can be derived from a shortening of the incubation time or a change in the temperature. While the absolute value of the blank can be reduced considerably, it remains substantially the same in relation to the
reducing power of steroids. The low pH of the reagent, which is responsible for hydrolysis of the cellulose, cannot be raised materially without seriously affecting the sensitivity. The latter depends on the formation of the yellow arsennomolybdate complex which requires a large excess of acid.

In the experience of the author, variations of the blank value and serious disagreement between duplicate determinations are largely due to contamination of the chromatogram strips with extraneous reducing matter. It is therefore apparent that a minimum of handling of the paper strips and scrupulous cleanliness on the bench and in the laboratory atmosphere are essential for satisfactory operation.

In spite of its inherent weaknesses, the arsennomolybdate method is capable of furnishing valuable information on the composition of adrenal extracts. It is hoped eventually to correlate the results obtained by quantitative chromatography with those of biological assays, thus enabling an estimate to be made of the potency, as well as the steroid distribution, of a particular extract.

REFERENCES


Studies in Rhodopsin

6. REGENERATION OF RHODOPSIN

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(Received 27 June 1952)

The history of visual pigments has been reviewed on numerous occasions, e.g. Collins & Morton (1950) and Wald (1951). Under the action of light the rhodopsin content of the eye decreases to an extent depending on time and intensity, whereas in darkness it gradually approaches a maximum. The photochemical destruction ('bleaching') is, however, normally incomplete (Lythgoe, 1940) as rhodopsin is found in light-adapted eyes. The regeneration of rhodopsin, in terms of dark adaptation in the living eye, approaches completion in 30 min. and is almost complete in 45 min. Both vitamin A deficiency (Hecht, Hendley, Frank & Haig, 1946; Medical Research Council, 1949) and anoxia (Hecht & Mandelbaum, 1940) are known to increase the time necessary for dark-adaptation. Collins (1951) submitted a brief communication to the Biochemical Society reporting work on regeneration of rhodopsin. These experiments used as starting points those of Zewi (1939) in his important quantitative extension of the pioneer work of Kühne (1878). In the present work in vitro preparations of eye tissues have been substituted for excised frog eyes, with exposed optic cups, as used by Zewi. Regeneration of rhodopsin might be either an endergonic or exergonic process. If the latter were the case the reaction would be spontaneous, while if the former were true it would have to be coupled to

SUMMARY

1. A method is presented for the quantitative evaluation of adrenal extracts in which the mixture of cortical steroids is resolved into its components by paper chromatography and the chromatogram is incubated with arsennomolybdate reagent. Steroids containing a ketol side chain or having an αβ-unsaturated 3-keto structure quantitatively reduce the reagent to molybdenum blue.
2. The blue zones of the developed chromatogram corresponding to the various cortical steroids are eluted and the blue complex is determined in a photoelectric colorimeter.
3. About 5-100 µg. of reducing steroid can be determined with an accuracy of ±10%.
4. The reducing steroids are determined in two synthetic mixtures and two adrenal extracts.

The author wishes to thank Dr Karl Folkers of Merck and Co., Inc., U.S.A., for the generous gift of some of the steroids used in this investigation; and the Directors of Allen and Hanburys Ltd., for permission to publish this communication.