CORTICOSTEROIDS IN URINE OF NORMAL PERSONS DETERMINED BY PAPER CHROMATOGRAPHY*

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The presence in human urine of small amounts of material with adreno-cortical activity is well established (1, 2). The chemical characterization of this urinary material by the classical methods used for the steroids in adrenal glands has been technically difficult. In the six reported instances (3-8) in which corticoids have been isolated and identified by these procedures, extracts of large volumes of urine containing quantities of corticosteroids of the order of several mg. were employed. These methods, furthermore, cannot be routinely used in the isolation of the corticosteroids present in conveniently small aliquots of urine.

A paper chromatographic method for the separation of microgram quantities of adrenocortical steroids has already been reported (9). In the preceding paper (10), the value of this procedure has been demonstrated in the separation of the components in small quantities of adrenal cortex extract and a systematic method for the identification of these corticoid components, embracing both chromatographic and spectrophotometric techniques, has been described. The application of this method to the analysis of urinary extracts for their minute content of corticoids is complicated by the presence of very much larger quantities of pigments and other contaminating materials. The procedure devised for urine extracts is, consequently, more lengthy than that used in the analysis of adrenal extracts.

This paper describes a method which requires relatively small samples of urine and can, therefore, be used in extensive studies of corticoid excretion patterns. Also given are the results of the analyses of urine specimens, each representing 3 days excretion, from normal persons, in which the presence of 17α-hydroxycorticosterone (Kendall's Compound F) and of 11-dehydro-17α-hydroxycorticosterone (cortisone) was demonstrated, as reported in preliminary communications (11, 12).

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Urine Collection and Extraction—Individual urine specimens representing 72 hours excretion were collected from twelve normal persons, six males and six females. These specimens, with the exceptions indicated below, were separately analyzed throughout. The specimens were kept in a refrigerator during collection with a small amount of toluene added as a preservative. Thymol could not be used for this purpose, since it was found to increase the bulk of the extract and to interfere with the chromatographic fractionation. Anhydrous sodium sulfate, technical grade, was added with stirring to give a concentration of 70 gm. per liter, in order to reduce emulsion formation during the extraction. The urine was acidified to pH 1 against the hydrogen electrode with 50 per cent sulfuric acid (volume per volume). The specimens were then continuously extracted with U.S.P. ether for 48 hours in apparatus designed to accommodate urine volumes of 3 to 5 liters.

Each ether extract was evaporated to dryness. The residue was dissolved in 200 ml. of U.S.P. chloroform and the chloroform solution washed with 25 ml. portions of 0.1 N sodium hydroxide. The alkali washings were repeated until no more pigment was removed from the chloroform phase, a minimum of four washings being carried out in all cases. The chloroform solution was then washed with two 25 ml. portions of 0.1 HCl hydrochloric acid and sufficient 25 ml. portions of distilled water to remove all traces of acid. Each alkali, acid, and water wash was reextracted with one 15 ml. portion of chloroform which was added to the original chloroform solution before the next wash was carried out. The chloroform solution was dried with c.p. anhydrous sodium sulfate and decanted through sintered glass. The salt, transferred to the filter, was washed three times with 20 ml. portions of chloroform which were added to the original solution. This neutral extract was evaporated to dryness and the residue ultimately transferred quantitatively to a 10 × 75 mm. test-tube with U.S.P. methanol. The methanol was evaporated and the tubes stoppered and stored in a refrigerator until further analyzed.

The scheme of paper chromatographic fractionation, purification, and analysis to which these extracts were then subjected is presented in the flow sheet in Fig. 1.

Fractionation of Neutral Extracts—The neutral extracts were divided into three fractions, A, B, and C, by the paper chromatographic technique and apparatus described previously (9, 10). The papers were of the same shape as those described in the preceding article, except that a slot 0.5 cm. wide
72 Hour Urine Specimen

Neutral Extract

Chromatographed in B-F 48 Hours

Overflow
0-5 Hrs.
Fraction A
Chromat.

Discard pigment

11-D.O.C.
Area eluted
HI04 oxid.
Chromat.
Butanol-NH4OH
Ascending
Discard pigment

Δ4,3-Keto-Etiocholenic Acid eluted
Chromat.
Butanol-NH4OH
Sample on 0.5 cm. area
Color test

Overflow
5-48 Hrs.
Fraction B
Chromat.

Repeat if nec.
Discard pigment

Rest of paper eluted
Chromat.
B-F Narrow strip
Strip divided lengthwise
Color test α-Ketols

Individual compds. eluted for further tests

Paper dried

Elute with methanol=Fraction C
Chromat.
T-PG. Narrow strip
Strip divided lengthwise
Color tests α-Ketols and special reactions
Individual steroids eluted for further tests

Note: B-F = Benzene-Formamide (Methanol-diluted)

B-F* = Benzene-Formamide (undiluted)

T-PG. = Toluene-Propylene Glycol (Methanol-diluted)

Fig. 1. Analysis of urinary corticoids by paper chromatography
near one lateral edge was cut from the bottom to a point 2 cm. above the starting line so as to give a 1.0 cm. wide pilot limb and 15.5 cm. wide main limb.

The fractionation of the urine extracts was similar to that used for adrenal cortex extracts (10) with certain modifications. The papers were impregnated with a 50 per cent solution (volume per volume) of formamide in absolute methanol. The bulky urine extracts could be applied in a narrower zone along the starting line when formamide rather than propylene glycol was used as the impregnating solvent. The extracts were applied to the main limb and 25 γ of cortisone to the pilot limb. Development with formamide-saturated benzene (350 ml. in the reservoir trough) was carried out for 48 hours and the overflow collected in two separate beakers. The solvent collected during the first 5 hours constituted Fraction A. That collected during the next 43 hours constituted Fraction B. The paper was dried with a warm air fan for 6 hours and the pilot limb cut off and treated with triphenyltetrazolium chloride (TPTZ) reagent (9). The presence of the spot of cortisone on the lower one-third of the strip served as an indication that the rate of movement of steroids during the fractionation procedure was comparable from run to run. A pigmented zone which never extended more than 1.5 cm. below the starting line of the main limb was discarded by cutting immediately below it. The remainder of the main limb was eluted to give Fraction C.

Fractionation of mixtures of the six active corticosteroids by this modified procedure gave the same distribution of compounds as that obtained with the use of the toluene-propylene glycol system (10).

Purification and Analysis of Fractions—In order to detect the very small amounts of individual corticoids, it was necessary to perform the analytical chromatograms on paper strips as narrow as possible. Since the fractions obtained at this point were too bulky to be applied to strips sufficiently narrow, it was necessary to purify them by chromatography on wide papers before the analytical runs. The purification steps will be described along with the analytical conditions for each of the three fractions.

Fraction A—This fraction, the bulkiest and most highly pigmented of the three, was analyzed for the presence of 11-desoxycorticosterone. The entire fraction was applied to a paper impregnated with undiluted formamide of the dimensions and shape given above. Approximately 25 γ of 11-desoxycorticosterone were applied to the pilot and the chromatogram was developed with benzene for 4 hours. The paper was dried and the pilot treated with TPTZ. The portion of the main limb corresponding to the position of 11-desoxycorticosterone was removed by cutting along lines 2 cm. above and 2 cm. below the limits of the pilot spot. The remainder of the paper, usually highly pigmented, was discarded. The 11-desoxycorticosterone zone was eluted and the eluate evaporated to dryness.
The residue obtained was still too pigmented and bulky to permit chromatographing on strips sufficiently narrow to allow detection of very small amounts of 11-desoxycorticosterone. In order to circumvent this difficulty the residue was treated with periodic acid (10), thereby oxidizing any 11-desoxycorticosterone to Δ⁴-3-ketoetiocholenic acid, a compound of markedly different chromatographic properties.

It was now possible to separate any etioacid from the bulk of contaminants by paper chromatography by using the system, n-butanol:10 per cent aqueous ammonium hydroxide (10). Dry filter paper sheets, 28 × 42 cm., were prepared with a starting line drawn parallel to and 6 cm. from one of the narrow edges. Marks were placed along the starting line 7 cm. from each side edge, thus designating a central 14 cm. long segment of the line to which the residue was evenly applied. Several 50 μl. portions of absolute methanol were used in the quantitative transfer of the residue to the filter paper. Approximately 20 μg samples of pure Δ⁴-3-ketoetiocholenic acid were applied to points on the starting line 2 cm. from each side edge. The blank spaces 5 cm. wide separating the reference compound from the urinary material were sufficient to prevent any cross-diffusion during development. The papers were stapled to form cylinders and were developed with the butanol system in the manner outlined (10).

After drying the paper, strips containing the reference compound were obtained by cutting along lines parallel to and 4 cm. from each side edge. The reference strips were exposed to hydrochloric acid fumes for 1 to 2 minutes and then were sprayed lightly with the iodine reagent (9), whereupon a blue spot appeared at the position of the etioacid \( R_f = 0.60 \). The corresponding area of the untreated central portion of the chromatogram was cut out and eluted with methanol. The methanol was evaporated, leaving a residue of very small bulk with a slight brownish color. It was then possible to apply quantitatively this small quantity of material to a circular area about 0.5 cm. in diameter on the starting line of another chromatogram, which again carried reference samples of the authentic etioacid. Development was carried out as in the preceding run. This time the entire sheet was treated with the iodine reagent.

In a model experiment 20 μg of pure 11-desoxycorticosterone were added to one-half of a 144 hour urine extract. Each of the two aliquots was treated by the above procedure. In the final chromatogram of the aliquot with added 11-desoxycorticosterone, a blue spot was found with an \( R_f \) value identical to that of the reference sample of etioacid. This spot was absent in the chromatogram of the other aliquot, a finding common to all of the urinary specimens we have studied (see below).

Fraction B—The residue obtained on evaporation of the benzene was chromatographed on the main limb of a 17 cm. wide paper. Approxi-
approximately 25 γ of 11-dehydrocorticosterone were applied to the pilot limb.2 The paper was developed with either benzene-formamide or toluene-propylene glycol (polar solvent diluted with an equal volume of methanol for impregnation) for 5 hours with 350 ml. of solvent in the reservoir. At the end of this period it was dried, and the pilot limbs were cut off and treated with TPTZ. The presence of 11-dehydrocorticosterone, the fastest moving C21O4 corticoid, on the pilot was taken as evidence that none of the steroids belonging in Fraction B had run off the main limb. A heavily pigmented zone which never extended more than 3 cm. below the starting line was usually seen on the main limb. The paper was divided along a line just below this zone, which was discarded. Model chromatograms with pure compounds mixed with this pigmented material showed that neither 11-desoxy-17α-hydroxycorticosterone, corticosterone, nor 11-dehydrocorticosterone was ever present in this discarded area. The remainder of the paper was eluted with methanol and the solvent evaporated.

The residue obtained at this point was still too bulky and pigmented, at times, for final analysis. Overloading of the preceding chromatogram may have been the responsible factor. When the above procedure was repeated, however, sufficient purification was usually obtained, more pigment being retained in the upper zone. In one case (C. W.) large quantities of other pigments were encountered close to the positions of the known C21O4 corticoids. Since they could not be eliminated, the final analysis could not be carried out.

For the final analytical run a paper 42 cm. long, consisting of two 1.0 cm. wide limbs, was prepared. The purified residue was quantitatively transferred to one of the limbs and approximately 20 γ each of corticosterone and 11-dehydrocorticosterone2 were applied to the other limb. When residues representing excretion periods of other than 72 hours were analyzed, the limbs to which they were applied were made of a width corresponding to 1.0 cm. for every 72 hours of extract. The chromatogram was developed with the same solvent systems and under the same conditions used in the purification steps. After drying, a strip 0.25 cm. wide was cut lengthwise from the limb containing the urinary residue. This and the reference limb were treated with TPTZ and compared. If a spot was found on the treated urinary strip, the corresponding area of the other strip was cut out and eluted, and the material saved for further examination.

Fraction C—The residue obtained on evaporation of the methanol, still too bulky and pigmented for final analysis, was applied to the main limb.

We have also used for this purpose Fraction B obtained from commercial aqueous adrenal cortex extracts (10). Aliquots of adrenal Fraction B corresponding to 0.5 ml. of the original aqueous preparation were used in the pilot.
of a 17 cm. wide paper. Approximately 20 \( \gamma \) of cortisone\(^3\) were applied to the pilot limb. The chromatogram was developed with toluene-propylene glycol (methanol-diluted) for 48 hours with 350 ml. of solvent in the reservoir. The paper was dried and the pilot limb treated with TPTZ. The presence of cortisone on the pilot was again taken as evidence that no \( \text{C}_\text{H}_\text{O}_\text{S} \) steroid had run off the main limb. Additional pigment which was retained in the area close to the starting line was discarded and the rest of the main limb eluted.

For the analytical chromatogram a 42 cm. long paper with two limbs was prepared. The pilot limb, to which approximately 20 \( \gamma \) of cortisone\(^3\) were applied, was made 1.0 cm. wide. The limb for the urinary residue was of a width corresponding to 1.0 cm. for each 24 hours of extract analyzed. This paper was also developed with the toluene system under the conditions given above. After drying, two 0.25 cm. wide strips were cut lengthwise from the urinary limb. One strip and the pilot limb were treated with the TPTZ reagent and the positions of the spots compared. The other strip was treated with the iodine and sulfuric acid reagents as described below. Areas of the remaining urinary limb corresponding to those of the spots on the TPTZ-treated urinary strip were cut out and eluted.

**Identification of Isolated Materials**—Our purpose in this work was to identify the corticosteroids in the separate specimens. Because the amounts of the principal compounds obtained in each case were very small, only a few of the procedures described for the final identification of adrenal extract components (10) could be used. These are listed below. In some specimens additional compounds were isolated in quantities too small for identification.

**Color Reactions**—Alkaline silver nitrate (9) was at first used to detect \( \alpha \)-ketols on the chromatograms. More recently the TPTZ reagent, as indicated above, was used. The iodine reagent was used upon areas of developed chromatograms corresponding to the position of cortisone. Concentrated sulfuric acid (10) was used upon areas of developed, untreated chromatograms corresponding to the position of known 11-\( \beta \)-hydroxy cortisoloids or of 11-desoxy-17\(\alpha\)-hydroxy corticosterone.

**Ultraviolet Absorption Spectrum**—An aliquot of the eluted urinary material corresponding to at least 20 to 30 \( \gamma \) was dissolved in 3 ml. of absolute methanol and its spectrum determined from 220 to 270 \( \mu \)\(\text{m} \).

**Mixed Chromatograms**—Due to the small amounts of material, the procedure previously described (10) was modified so that a paper with two

\(^3\) Fraction C, obtained from commercial aqueous adrenal extracts (10), was also utilized for this purpose. Aliquots of this fraction corresponding to 0.7 ml. of the original preparation were used in the pilot.
CHROMATOGRAPHY OF STEROIDS IN URINE

limbs, each 0.5 cm. wide, was used. An aliquot of the unknown corresponding to about 15 γ and an equal amount of the known were applied at the starting line of one of the limbs. A second aliquot of the known was placed on the other limb.

Preparation of Acetates and Propionates—These were prepared (10) from aliquots of the eluted compounds representing about 20 to 25 γ of steroid. Mixed chromatograms were carried out with modification as described.

RESULTS AND DISCUSSION

The α-ketolic compounds found in the urine specimens of the twelve normal subjects are listed in Table I. It should be pointed out that steroids originally present in the urine as glucuronide conjugates probably were not hydrolyzed by the procedure employed and were not, therefore, present in the neutral extracts.

11-Desoxycorticosterone (as Δ₄-3-ketoetiocholenic acid) was not found in Fraction A of any of the twelve specimens. In the six specimens analyzed separately (R. B., E. K., H. N., R. H., A. S., L. Z.), the negative result indicated that, if free 11-desoxycorticosterone was normally excreted, it was in amounts less than 5 γ per day (the iodine reagent detects a minimum of 15 γ under the conditions described (9)). In the remaining six cases the subfractions in which the etioacid might be found were pooled and one final analytical chromatogram was run with the combined material representing 18 days excretion. If non-conjugated 11-desoxycorticosterone was excreted by these subjects, it was, therefore, in quantities under 0.8 γ daily. The low solubility of 11-desoxycorticosterone in water suggests that, if this steroid is normally excreted, it probably would be as the glucuronide or some other water-soluble conjugate.

No α-ketol was found in Fraction B of eight specimens. In the remaining four, small quantities of TPTZ-reducing material were noted on the final chromatograms. In two instances (R. B., H. N.) material was seen at positions near those of corticosterone and 11-dehydrocorticosterone, designated Subfractions B-2 and B-3, respectively. In the other two (A. S., F. K.) the spot, Subfraction B-2, was seen again, as well as another, designated Subfraction B-1, at a position somewhat above that of corticosterone. In none of these specimens was sufficient material present for characterization.

All specimens contained, in Fraction C, two compounds which were identified as 17α-hydroxycorticosterone (Kendall’s Compound F) and 11-dehydro-17α-hydroxycorticosterone (Kendall’s Compound E or cortisone). The former compound was identified on the basis of these data: (1) It

Unidentified urinary materials, designated subfractions for convenience, should not be considered synonymous with subfractions described in the preceding paper (10).
Table I

Non-Conjugated Urinary Corticoids in Normal Individuals

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Fraction A</th>
<th>Fraction B</th>
<th>Fraction C</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. B.</td>
<td>30</td>
<td>M</td>
<td>72 hr.</td>
<td>B-2 1+</td>
<td>Compound F 1+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B-3 4+</td>
<td>&quot; E 3+</td>
</tr>
<tr>
<td>A. C.</td>
<td>25</td>
<td>&quot;</td>
<td>18 day‡</td>
<td>None</td>
<td>&quot; F 1+</td>
</tr>
<tr>
<td>E. K.</td>
<td>52</td>
<td>&quot;</td>
<td>72 hr.</td>
<td>(36 hr. ali-</td>
<td>&quot; E 4+</td>
</tr>
<tr>
<td>H. N.</td>
<td>25</td>
<td>&quot;</td>
<td>72 &quot;</td>
<td>B-2 1+</td>
<td>Fraction lost</td>
</tr>
<tr>
<td>R. S.</td>
<td>27</td>
<td>&quot;</td>
<td>18 day‡</td>
<td>None</td>
<td>&quot; E 3+</td>
</tr>
<tr>
<td>A. Z.</td>
<td>26</td>
<td>&quot;</td>
<td>18 &quot; ‡</td>
<td>&quot; C-1 1+</td>
<td></td>
</tr>
<tr>
<td>H. C.</td>
<td>27</td>
<td>F</td>
<td>18 &quot; ‡</td>
<td>&quot; C-1 1+</td>
<td></td>
</tr>
<tr>
<td>R. H.</td>
<td>23</td>
<td>&quot;</td>
<td>72 hr.</td>
<td>&quot; C-1 1+</td>
<td></td>
</tr>
<tr>
<td>F. K.</td>
<td>26</td>
<td>&quot;</td>
<td>18 day‡</td>
<td>B-1 1+</td>
<td></td>
</tr>
<tr>
<td>A. S.</td>
<td>26</td>
<td>&quot;</td>
<td>72 hr.</td>
<td>B-1 1+</td>
<td></td>
</tr>
<tr>
<td>C. W.</td>
<td>32</td>
<td>&quot;</td>
<td>18 day‡</td>
<td>Not analyzed</td>
<td></td>
</tr>
<tr>
<td>L. Z.</td>
<td>25</td>
<td>&quot;</td>
<td>72 hr.</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

* 72 hour aliquot unless otherwise noted.
† 24 hour aliquot.
‡ Fractions pooled.
§ = 10 to 15 γ; 1+ = 15 to 20 γ; 2+ = 20 to 25 γ; 3+ = 25 to 30 γ; 4+ = 30 to 40 γ.

had a positive reaction for an α-ketol group with TPTZ or alkaline silver reagents. (2) It had yellow-green color with green fluorescence on treatment with concentrated sulfuric acid, typical of 17α-hydroxycorticosterone.
The ultraviolet absorption spectrum had a maximum at 240 m\(\mu\) characteristic of \(\alpha,\beta\)-unsaturated ketosteroids. Its chromatographic position was that of 17\(\alpha\)-hydroxycorticosterone. This was borne out by the behavior in mixed chromatograms with authentic 17\(\alpha\)-hydroxycorticosterone. The acetate and propionate each behaved like the respective ester of authentic 17\(\alpha\)-hydroxycorticosterone in mixed chromatograms. Cortisone was identified from these data in each case: (1) positive reactions with either TPTZ or alkaline silver reagents; (2) intense blue color with the iodine reagent, a reaction given only by 11-dehydro-17\(\alpha\)-hydroxycorticosterone of the many \(\alpha\)-ketolic steroids tested (8); (3) maximum ultraviolet absorption at 240 m\(\mu\); (4) chromatographic behavior identical to that of authentic cortisone, as shown by mixed chromatograms; (5) the acetate and propionate derivatives each behaving like the respective authentic ester in mixed chromatograms.

By rough matching of the size and intensity of the spots of these urinary compounds with those given by known amounts of pure 17\(\alpha\)-hydroxycorticosterone and 11-dehydro-17\(\alpha\)-hydroxycorticosterone, it was estimated that all subjects excreted between 15 and 40 g of each compound daily. In each specimen the amount of cortisone was slightly the greater. In six instances these estimates were checked by measurement of the optical density of the eluted urinary compounds at 240 m\(\mu\) (10). It is of interest that the combined amounts of cortisone and Compound F excreted by these subjects correspond closely to the amounts reported by Venning and Kazmin (2), who found that the total glycogenic activity of extracts of urine of normal persons corresponded to between 25 and 85 g of active C\(_{21}\)O\(_6\) corticoid per day.

In six specimens a third material, Subfraction C-1, which moved slower than 17\(\alpha\)-hydroxycorticosterone, was found in Fraction C. In no case was the amount excreted greater than 20 g daily (visual estimate of the spots). The position of this material on the chromatograms indicated that it was more polar than 17\(\alpha\)-hydroxycorticosterone. On the basis of our work with adrenal cortex extracts (10), it was suspected that this material was not homogeneous. The small amounts obtained prevented its fractionation and further attempts at its identification.

The isolation of biologically active adrenocortical steroid from human urine was first reported by Mason and Sprague (3). These investigators isolated 17\(\alpha\)-hydroxycorticosterone from the urine of a patient with Cushing’s syndrome, whose excretion of this steroid was of the order of several mg daily.

Schneider (5, 6) has reported the isolation of cortisone, pregnane-17\(\alpha\),21-diol-3,11,20-trione, pregnane-3\(\alpha\),17\(\alpha\),21-triol-11,20-dione, and 17\(\alpha\)-hydroxycorticosterone from pooled extracts of urine collected during
the daytime from normal males. He extracted 1000 liters of urine and isolated 55.3 mg. of cortisone and 6 mg. of the related 3α-hydroxyproginate. The quantities of the other compounds were not recorded.

**SUMMARY**

A method utilizing paper chromatography is described for the analysis of adrenocortical steroids in urine extracts. Specimens of urine from twelve normal persons representing relatively small periods of excretion were separately analyzed. All the subjects excreted both cortisone and 17α-hydroxycorticosterone in amounts between 15 and 40 μg of each daily. In some cases smaller amounts of uncharacterized material, probably steroids of the C21O4 and C21O6 series, were found. None of the specimens could be shown to contain 11-desoxycorticosterone.

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