METHODS FOR THE ESTIMATION OF GROUPED URINARY STEROIDS BY QUANTITATION AS 17-KETOSTEROIDS

By

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ABSTRACT

Methods for the estimation of urinary total 17-ketosteroids and 17-hydroxycorticosteroids, both total and 11-deoxy and 11-oxy fractions are described. It was found that for the hydrolysis of 17-ketosteroid conjugates in a boiling water bath better results were obtained by boiling the material for 7 minutes with hydrochloric acid 4 mol/l than for 35 minutes with hydrochloric or sulphuric acid 1 mol/l. The 17-hydroxycorticosteroids were estimated using the reaction sequence – borohydride reduction, periodate oxidation, basic hydrolysis, partition chromatography and quantitative estimation of the 17-ketosteroids produced. Basic hydrolysis of the formates produced by periodate oxidation was complete at pH 13, but not at pH 9. Quantitation of the 17-ketosteroids was carried out by a modified Zimmermann reaction, extraction of unionised colourless forms of the reaction products, conversion of these into quinoid forms and measuring the extinction at 530 nm. KOH 4 mol/l, methanol as the reaction medium and incubation for 35 minutes at 13–15°C were found to be optimal for colour development. A two phase system consisting of a water phase with KOH concentrations of less than 0.16 mol/l and a pure dichloromethane phase produced optimal conditions for the extraction of the unionised stable reaction products of androsterone, aetiocholanolone, dehydroepiandrosterone and 11-hydroxy-aetiocholanolone. The concentration of the methanol in the water phase was 20% (v/v). Excellent specificity was obtained for the technique used, when evaluated by the absorption spectra for urinary 17-ketosteroids and by comparing the values obtained with and without purification by partition chromatography. It is concluded that the methods are simple, reliable and suitable for routine analysis.
Total neutral 17-ketosteroids in urine are often estimated according to the well-known principle: acid hydrolysis, isolation of the liberated 17-ketosteroids and quantitation by a Zimmermann reaction.

The 17-hydroxycorticosteroids in urine (this term covers 17-hydroxy-C-21 steroids with either a 20-oxo or a 20-hydroxyl group) are estimated in many methods according to the principle developed by Norymberski & Stubbs (1953) and Appleby et al. (1955): borohydride reduction of all the oxo groups including the 17-ketosteroids in situ, oxidation of 17-hydroxycorticosteroids (»17-ketogenic« steroids) into 17-ketosteroids, isolation of the generated 17-ketosteroids and estimation by a Zimmermann reaction. The oxidation is carried out using bismuthate, which together with the acid hydrolysis produces different artifacts (Few 1961; Pesonen 1963); these limit the usefulness of the method for accurate analytical work. Few (1961) subsequently found that these disadvantages could be overcome by the use of periodate as the oxidizing agent. The reaction sequence – borohydride reduction, periodate oxidation, mild basic hydrolysis – converts the 17-hydroxycorticosteroids to 11-OH-aetiocholanolone (11-OH-AE) and aetiocholanolone, two 17-ketosteroids with a known chromogenicity in the Zimmermann reaction. In addition these can be separated by a simple partitioning chromatographic technique. It is thus possible to estimate the urine metabolites of cortisol – 11-oxy-17-hydroxycorticosteroids – without any interference from pregnanetriol and other urine metabolites of 11-deoxy-17-hydroxycorticosteroids.

The last analytical step for total neutral 17-ketosteroids (Tot. 17-KS) and for 17-hydroxycorticosteroids (17-OHCS) – quantitation by a Zimmermann reaction – is common to both groups of steroids. The analytical specificity in both cases becomes a problem when the analyses, of necessity, must be simplified for routine use.

Simplified methods are described in this study for the estimation of Tot. 17-KS, Tot. 17-OHCS, 11-oxy-17-OHCS and 11-deoxy-17-OHCS. The following analytical steps have been the object of particular study: acid hydrolysis in the estimation of Tot. 17-KS. Separation of 17-OHCS (Few 1961) and in this respect, the basic hydrolysis of 17-ketosteroid products of the periodate oxidation. The quantitation of 17-ketosteroids by a modified Zimmermann reaction and extraction of the specific unionised chromogens require particular consideration with regard to the specificity in the section Colorimetry. Finally the reliability of the methods is evaluated.

MATERIALS AND METHODS

Tubes 35–40 ml, NS-24 and 12 ml, NS-14 with polythene stoppers.
Chromatography tubes diameter 10–12 mm, length approx. 250 mm, with teflon stopcock.
Mechanical shaking apparatus.

Vortex Mixer.

1,2-Dichloroethane, reinst Merck was used without purification.

Celite 545 was purified by letting it stand overnight with three times its weight of HCl 6 mol/l, washing with water until free of chloride and drying at 100° C.

Methanol p. a. Merck was used without purification.

Petroleum ether British Drug House, the fraction with a boiling range 60–80° C, free from aromatic hydrocarbons, was used without further purification, but other petroleum qualities may be used when sufficiently purified.

Potassium hydroxide 6.0 mol/l in methanol was prepared by dissolving a surplus of KOH pellets in methanol, cooling to room temperature, standing overnight and decanting. Methanol was added until the concentration of KOH was in the range of 5.95–6.05 mol/l. A slight turbidity of the solution is of no importance.

m-Dinitrobenzene 20 g/l in methanol. 1,3-dinitrobenzene, Merck was used without further purification.

Benzene, analytical reagent grade was washed several times with concentrated sulphuric acid and then with purified water until neutral, then dried and redistilled.

Solvents used for the celite partition columns were prepared by mixing 270 ml petroleum ether, 30 ml benzene, 50 ml methanol, and 60 ml water and allowing the mixture to stand until clear. The upper layer was used as the mobile phase and the lower as the stationary phase. Other reagents used were of analytical reagent grade.

Steroids used:

1) Androsterone = 3α-hydroxy-5α-androstan-17-one
2) Aetiocholanolone = 5α-hydroxy-3β-androstan-17-one
3) Dehydroepiandrosterone (DHEA) = 3β-hydroxy-androst-5-en-17-one
4) DHEA-sulphate, sodium salt
5) Androstenedione = Androst-4-ene-3,17-dione
6) Dihydrotestosterone = 5α-androstan-17β-ol-3-one
7) Androstenedione = Androst-4-ene-3,11,17-trione
8) Tetrahydrocortisol = 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one
9) 11-hydroxy-aetiocholanolone (11-OH-AE) = 3α,11β-hydroxy-5β-androstan-17-one
10) 11-keto-aetiocholanolone = 3α-hydroxy-5β-androstane-11,17-dione

Items 1–6: Sigma Chemical Company

Item 7: Calbiochem (California Corporation for Biochemical Research)

Items 8–10: Zori Pharmaceutical & Chemical Industrial Co. Ltd.

Urine Samples

24 h specimens of urine were used. When the daily volume was <1 l it was made up to that volume with water except for children under 12 years of age. If the urine sample was not subjected to immediate analysis it was stored at 4° C or less. Stored urine was heated to 40–50° C for a few minutes and shaken vigorously before analysis.

Total 17-KS

All samples were assayed in duplicate. 5.0 ml of hydrochloric acid 12 mol/l were added to 10.0 ml of urine in 40 ml tubes. The tubes were loosely stoppered, placed in a boiling water bath for exactly 7 minutes and then immediately cooled in cold water. A reagent blank was obtained by treating 10 ml water as a urine sample.
10.0 ml 1,2-dichloroethane were added to the samples which were then vigorously shaken in a mechanical shaking apparatus for 6 minutes. After brief centrifugation, the water phase was removed, the crude dichloroethane extract passed through filter paper and the extract shaken with 10–20 NaOH pellets for 3 minutes. After repeated filtration, 3.00 ml of the purified dichloroethane extract were evaporated to dryness in 40 ml tubes in a slowly boiling water bath. 30 µg of androsterone evaporated from an ethanol solution served as the standard. The evaporated extracts were stored in a desiccator containing silicagel until colorimetry. (See the section Colorimetry).

**Total and Fractionated 17-OHCS**

All urine samples were tested for glucose using Clinistix®. If a positive reaction was obtained, the glucose concentration was estimated semiquantitatively by the Clinitest® method. The analyses were carried out on two different urine dilutions with glucose concentrations < 20 g/l; these were chosen according to the glucose concentration:

<table>
<thead>
<tr>
<th>Glucose g/l</th>
<th>Vol. urine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>Vol. urine</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>undil. and 1+1</td>
</tr>
<tr>
<td>Vol. water</td>
<td>—</td>
</tr>
</tbody>
</table>

Both dilutions must give the same results.

With glucose concentrations > 20 g/l the urine was extracted thrice with 1/2 vol. ether/ethanol 3:1 after the addition of sulphuric acid to pH 2 and 50 g ammonium sulphate per 100 ml urine. The total ether/ethanol extracts were evaporated in vacuum at a maximum of 40°C. Water was added to the residue in sufficient quantities to produce the original volume. The analyses were then performed as with normal samples.

All samples were assayed in duplicate except for the fractionating step. A reagent blank was obtained by treating 10 ml of water as a urine sample.

10.0 ml of fresh urine in a 40 ml tube were adjusted to pH 7–8 by the addition of NaOH 4 mol/l and using indicator paper. 1.0 ml of freshly prepared sodium boro-hydrde solution, 100 g/l in NaOH 0.1 mol/l, was then added. After reduction for 1 h in a water bath at 37°C the surplus boro-hydrde was destroyed with 1.0 ml acetic acid 4 mol/l and standing for 15 minutes at 37°C. Excessive frothing during the reduction was controlled by spraying with ethanol. Oxidation was carried out by the addition of 4.0 ml NaIO₄ 100 g/l, adjustment to approximately pH 6.5 (6–7) adding approximately 10 drops of NaOH 4 mol/l, and incubation for 60 minutes in a water bath at 37°C. The basic hydrolysis of the formates was performed by the addition of 2.0 ml of NaOH 10 mol/l and further incubation for 15 minutes at 37°C. After cooling in cold water, extraction was made by shaking for 6 minutes in a shaking apparatus with 10.0 ml 1,2-dichloroethane and then vigorous centrifugation. During centrifugation a gel like layer was often formed between the dichloroethane phase and the upper urine phase. The urine phase could easily be removed, after which the extract was filtered through filter paper without transferring the gel layer to the filter. The remainder of the emulsified dichloroethane extract was freed by
shaking the tube and then passing its content through the filter. 3.00 ml of the extract for Tot. 17-OHCS and 5.00 ml for the fractionation were evaporated to dryness in 40 ml tubes in a slowly boiling water bath. The 5 ml extract was obtained by mixing the remaining extract from the tubes for the duplicate assays of the Tot. 17-OHCS which were always performed at the same time as the fractionation. 30 μg androsterone obtained by evaporation of an ethanol solution were used as the standard. The evaporated extracts were stored in a desiccator containing silicagel until colorimetry was carried out. (See the section Colorimetry).

Chromatography

1 g of celite was mixed with 1.0 ml of the stationary phase the latter being added in three portions of 0.4, 0.4, and 0.2 ml respectively, with rapid mixing after each addition. A small glass beaker and glass spatula were used for the mixing. This mixture was suspended in the mobile phase and transferred to the chromatography tube, the exit of which was plugged with glass wool. In addition, as much of the mobile phase was added, thus enabling the celite to be suspended by rapidly moving a glass plunger up and down. The column was then packed by gentle, but firm downward movements of the plunger. The surplus mobile phase was withdrawn so that the celite was only just covered by the mobile phase.

The residue from the dichloroethane extract was dissolved in 0.2 ml of benzene by vigorous shaking and transferred to the column with 3 X 1 ml of the mobile phase. 3 ml of the eluate were collected in a 40 ml tube marked A, 5 ml of the mobile phase were added to the column and an additional 5 ml of the eluate collected in tube A, which contained aetiocholanolone eluates. The column was then eluted with 10 ml of benzene, which were collected in a tube marked B, which contained 11-OH-AE. The eluates were evaporated in a slowly boiling water bath. The residue was washed down with a small amount of methanol, which was again evaporated.

Colorimetry

1 part m-dinitrobenzene 20 g/l in methanol was mixed with 2 parts KOH 6 mol/l in methanol during cooling at 0° C. The red reagent was kept at 0° C and used within 30 minutes. The tubes containing residues of 17-ketosteroid extracts, reagent blanks and standards were placed in a water-ethanol bath at 0° C, and 1.00 ml of the reagent was added to each tube. All the tubes were then stoppered and transferred to a water bath at 13-15° C for 35 minutes. Each tube was rotated for approximately 10 seconds on a Vortex mixer, moistening the tube but not the stopper. This was repeated to ensure complete solution of the residues. After 35 minutes all the tubes were cooled to 0° C, and 4.00 ml dichloromethane added. 4.00 ml of acetic acid 0.90 mol/l were added separately to each of the tubes during simultaneous rotation on a Vortex mixer. After stoppering, the rotation was continued for approximately 20 seconds. Phase separation was allowed to proceed at 0° C for a minimum of 5 minutes. 3 ml of the lower phases were removed and transferred to 12 ml tubes, which were stoppered and stored at 0° C. This procedure was very easily carried out using a pipette to which a two-way cock at the 3 ml mark was smelted, the one outlet of the cock being connected to a vacuum. All the samples were placed in a water bath at 15-20° C for a few minutes before photometry in order to avoid condensation during the measurement. 0.60 ml of freshly prepared KOH/methanol 60 mmol/l was added separately to each sample for colour development and the extinction read immediately at 530 nm using water as the reference. During the whole
procedure and particularly during the extraction and addition of the colour developer, direct light was avoided. A series should not comprise more than about 40 tubes.

**Calculation**

The extinction of the urine samples at 530 nm was termed E. For Tot. 17-KS E is the extinction when the reagent blank is subtracted. For Tot. 17-OHCS the reagent blank was only used to confirm the purity of the reagents (E<sub>530</sub> ≤ 0.01). D is the urine output per 24 h in litres. The constants in the calculation include the amounts of dichloroethane extract used, the molecular weights and relative colour equivalents for androsterone, aetiocholanolone and 11-OH-AE (Table 3).

\[
\begin{align*}
\text{Tot. 17-KS} \quad \text{mg/day} &= \frac{E \times D \times 10}{E_{\text{standard}}} \quad \text{as androsterone} \\
\text{Tot. 17-OHCS} \quad \text{mg/day} &= \frac{E \times D \times 12.5}{E_{\text{standard}}} \quad \text{as tetrahydrocortisol} \\
\text{11-deoxy-17-OHCS} \quad \text{mg/day} &= \frac{E_A \times D \times 6.26}{E_{\text{standard}}} \quad \text{as pregnanetriol} \\
\text{11-oxy-17-OHCS} \quad \text{mg/day} &= \frac{E_B \times D \times 7.48}{E_{\text{standard}}} \quad \text{as tetrahydrocortisol}
\end{align*}
\]

Chromatographic control:

\[
\frac{(E_A + E_B) \times 100}{E_{\text{Tot. 17-OHCS}}}
\]

if per ml dichloroethane extract was less than 85% then the analysis was repeated.

**RESULTS AND DISCUSSION**

**Total 17-KS**

It is generally agreed that 17-ketosteroids are almost entirely excreted as glucosiduronates and hydrogen sulphates in about the same amounts. The 17-ketosteroids are liberated by warm acid hydrolysis more or less quantitatively, depending on the conditions of the hydrolysis, but at the same time some artifacts are formed some of which do not give the Zimmermann reaction. The optimal conditions for the hydrolysis given in the literature vary considerably. A survey is given by Birke & Plantin (1954). As emphasized by amongst others Hamburger & Rasch (1948) the optimal conditions for a sample of urine are not necessarily optimal for other samples of urines, as different urines may contain varying amounts of acid labile 17-ketosteroid glucosiduronates and hydrogen sulphates. A one step hydrolysis must therefore be attempted in order to produce optimal conditions for an »average urine«. This has often been attempted by experiments with pooled urines in which an evaluation was made of the total yield of Zimmermann chromogens or investigating the effect of different conditions of hydrolysis on the quantitative
yield of individual 17-ketosteroids, estimated by chromatographic methods (Birke & Plantin 1954; Vestergaard & Clausen 1962).

A relatively high acid concentration, hydrochloric acid 4 mol/l in a hydrolysis mixture and a short period of hydrolysis, 7 minutes in a boiling water bath, is used in the method described here. These conditions have been chosen on the basis of hydrolysis and stability experiments with different urines, urinary 17-ketosteroid-glucosiduronates and DHEA sulphate, the latter representing a typical acid labile 17-ketosteroid. It was found for glucosiduronates that HCl 4 mol/l in the hydrolysis mixture with 5–10 minutes hydrolysis produced 40–50 % more Zimmermann chromogens than HCl 1 mol/l or H2SO4 1 mol/l for 20–40 minutes hydrolysis. With regard to DHEA sulphate it was found that hydrochloric acid 4 mol/l in an aqueous solution of DHEA sulphate with hydrolysis for 7 minutes, gave the same amount of Zimmermann chromogens as HCl 1 mol/l or H2SO4 1 mol/l with hydrolysis for 35 minutes. In both cases the amounts of Zimmermann chromogens liberated constituted approximately 80 % of the non acid treated DHEA sulphate standard. Hydrolysis of 18 different urines, using HCl 4 mol/l for 7 minutes gave 0–26 % (mean 10.4 %) higher values than using H2SO4 1 mol/l for 25 minutes. Finally examination of the absorption curves for the liberated Zimmermann chromogens showed no qualitative differences for the conditions of hydrolysis investigated.

From these results it is concluded that, in a one step hydrolysis procedure, a relatively high acid concentration and short period of hydrolysis time is on the whole better for an »average urine« than a low acid concentration and a long period of hydrolysis. The fact that artifact formation of some of the 17-ketosteroids increases with a higher acid concentration is well-known and can be demonstrated by chromatographic methods (Vestergaard & Clausen 1962). In the estimation of Tot. 17-ketosteroids this, however, is of less importance as the main artifacts have nearly the same colour equivalents as the original 17-ketosteroids. This can be seen in the recovery experiments (Table 6).

Extraction of the 17-ketosteroids liberated by the acid hydrolysis is carried out with 1,2-dichloroethane. Experiments on extraction showed that androsterone, aetiocholanolone, and DHEA could be extracted quantitatively from water or acid hydrolysed urine in one extraction, as stated under the method. Purification of the crude dichloroethane extract from urine is very easily accomplished by NaOH pellets (Drekter et al. 1950). Washing of the dichloroethane extract with NaOH solution and water does not improve the purity of the extract. Evaporation of the dichloroethane extract in a slowly boiling water bath is easily carried out, and it has not been possible to demonstrate any loss or transformation by thin layer chromatography of the Zimmermann chromogens, even after repeated evaporation of 17-ketosteroids dissolved in 1,2-dichloroethane.
Total and Fractionated 17-OHCS

The method described is in principle that used by Few (1961). Only important modifications are mentioned in the following.

Urine samples that have been stored in the cold should be heated to 40–50°C for some minutes before analysis, and carefully shaken before removal of the sample. This was seen to be necessary in several cases in order to obtain reproducible results from the analysis of urines that have been stored in a deep freezer.

Glucose positive urines with a glucose content of <20 g/l were analysed in two dilutions as a control to ensure that both dilutions were sufficient. The necessary dilution was found by experiments in which glucose was added to the urine. With glucose concentrations >20 g/l the steroid conjugates were extracted from the urine by ether/ethanole (Edwards & Kellie 1958). The dilution of the 24 h urine samples to 4 l as used by Few (1961) is only suitable for urines with a low glucose content. Metcalf (1963) recommended the use of larger amounts of borohydride and periodate for glucose positive urines, but this may, according to Few, lead to artifact formation (11-keto-aetiocholanolone).

Reduction. One hour in a water bath at 37°C was used for practical reasons rather than 2 hours at room temperature, as in the method of Few (1961). Ethanol spray for the frothing is more practical and less disagreeable than either ether or octanol. Amounts up to 1 ml of ethanol do not affect the analysis. The excess borohydride was destroyed by 1 ml of acetic acid 4 mol/l, which always produced a pH < 6.

Oxidation. The optimal pH according to Few (1961) is 6.5–7.0. As pH adjustment within such narrow limits using indicator paper in routine analyses is difficult, the influence of pH on the Tot. 17-OHCS values was studied in the interval 5–8. pH 5–7 gave similar values but pH > 7 gave considerably lower values. After adjustment to pH 6.5 with a narrow range indicator paper electrometric pH measurements showed that the actual pH values were always within the limits 6–7.

Basic hydrolysis. The object of adding NaOH after oxidation is to hydrolyse the formates from the oxidation. This hydrolysis is necessary before partition chromatography as the 11-OH-AE formates will otherwise falsely enhance the aetiocholanolone fraction. Few found that pH 9.0 produced complete hydrolysis. Our own investigations of urine extracts by thin layer chromatography showed that pH 9 does not ensure a complete hydrolysis of the formates even with a prolonged reaction time. At pH 12 or more the hydrolysis was complete, as the thin layer chromatography only showed two spots corresponding to aetiocholanolone and 11-OH-AE. The stability of DHEA, aetiocholanolone, 11-OH-AE, 11-keto-aetiocholanolone, and urinary 17-keto-
steroids obtained from Tot. 17-OHCS estimations was then studied under strong basic conditions. The steroids were incubated for 30 minutes at 37°C in NaOH 1 mol/l and in water for comparison. No loss of Zimmermann chromogens could be demonstrated. Therefore the basic hydrolysis was always performed in the routine assays by the addition of relatively large amounts of base – 2.0 ml NaOH 10 mol/l. Thus the basic hydrolysis is complete for all the urines, as the pH is then approximately 13.

Extraction. As mentioned under Tot. 17-KS estimation, 1,2-dichloroethane is suitable for the extraction of 11-deoxy-17-ketosteroids. 11-OH-AE can also be extracted almost quantitatively. 95 ± 3% of added 11-OH-AE could be recovered from water and from reduced, oxygenated and base hydrolysed urine. Intensive studies of the washing procedure on 21 different urines including washing with NaOH pellets, acetic acid, aqueous NaOH, sodium dithionite and water, demonstrated no difference between washed and non-washed extracts. A simple filtration of the dichloroethane extract is sufficient. The same results were obtained by Metcalf (1963) who used chloroform for the extraction.

Chromatography. Separation of the formed 17-ketosteroids into an aetiocholanolone and an 11-OH-AE fraction representing respectively 11-deoxy-17-OHCS and 11-oxy-17-OHCS was performed by partition chromatography on celite column. The following changes were made as compared with Few's (1961) technique:

The residue from the evaporated dichloroethane extract was dissolved in 0.2 ml benzene instead of 0.1 ml, which was found to be necessary in order to ensure that the 11-OH-AE was completely dissolved. Methanol instead of ethanol and 60 ml water instead of 50 ml for the preparation of the stationary and mobile phase were necessary in order to avoid tailing in the aetiocholanolone fraction. The use of petroleum ether, boiling range 60–80°C instead of 80–100°C produced a more rapid evaporation of the column eluates. It should be noted that a rapid distribution of the stationary phase on the celite and a rapid addition of the mobile phase is important in order to avoid evaporation, and hence changes in the composition of the stationary phase. In order to ensure that no Zimmermann chromogens were lost during the fractionation, an estimation of Tot. 17-OHCS was always performed at the same time, and the sum of the Zimmermann chromogens from the two fractions was compared with the value for the total estimation. In an analysis of 200 normal urines this sum showed an average of 92%o. The analysis was repeated if the value was lower than 85%o.

Colorimetry

The Zimmermann reaction in principle is a condensation reaction in a basic milieu between m-dinitrobenzene and the active 16-methylene group in the
17-ketosteroids. Other carbonyl groups with free $\alpha$-H atoms may also give
the reaction. The mechanism of the reaction has not been completely elucidated
but recent studies (Corker et al. 1962; Roy 1962; Neuhoeffer et al. 1961) have
demonstrated that a 17-ketosteroid reaction product can exist in *unionised*
colourless forms, soluble in chloroform, and as a violet *quinoid* form – the
actual Zimmermann colour compound having an absorption maximum at
520–530 nm. The quinoid form can only exist in the presence of a base, is
unsoluble in chloroform, but soluble in water/ethanol mixtures, the stability
in this solvent depending on the water and base content and on the temperature.

Numerous modifications of the Zimmermann reaction are based mainly on
purely empirical studies of the reaction conditions. Wilson & Nathanson (1945)
studied the importance of the ethanol and KOH concentrations in the reaction
medium, water/ethanol mixtures. Corker et al. (1962) found that organic bases,
amongst others tetramethylammoniumhydroxide, gave a greater sensitivity and
a lower blank value than KOH as used in conventional methods, for example
the Medical Research Council (M. R. C.) standard method (1963). Birchall &
Mitchell (1965) demonstrated on the other hand, that the modification of the
M. R. C. standard method described by Wilson (1954), where KOH and in-
cubation for 3 h at 0° C are used, is not improved by the use of organic bases,
neither with regard to the sensitivity and specificity nor to the blank values.
On the contrary, KOH should be preferred as a cheap, constant and com-
mercially available reagent. The same investigators also find that incubation
at 0° C instead of at 25° C does not produce any great improvement with
regard to specificity.

Relatively few investigators, amongst others Sheath (1959) and Beale et al.
(1962) have used methanol as the reaction medium. Methanol has according
to Beale et al. (1962) considerable advantages as compared to ethanol/water
mixtures, as a relatively high concentration of KOH can be obtained without
the addition of water. Thus good sensitivity, linearity between extinction and
amount of 17-ketosteroids together with low blank values and good specificity
is obtained. After some preliminary experiments with Beale's modification of
the Zimmermann reaction the advantages of the method could be confirmed
on the whole with the exception of the postulated specificity. The absorption
spectra for pure 17-ketosteroids showed considerable difference from the
spectra for urine extracts. It is generally agreed that a correction is necessary
for the unspecific chromogens. Different mathematical methods for example
the Allen correction have been used. Other investigators (Cohen 1944; 
Masuda & Thuline 1953; Werbin & Ong 1954; Rappaport et al. 1960; Peterson

* The term *unionised* is used for the not ionised aci-forms and the term *quinoid* for
the ionised base-forms of the Zimmermann reaction product. Each of the two forms
can be converted into the other one.
& Pierces 1960; Birchell & Mitchell 1965; James & Jong 1961) have extracted the Zimmermann chromogens with an organic solvent after colour development. Solvents such as ether, amyl alcohol, chloroform, 1,2-dichloroethane and dichloromethane have been used for this purpose.

It was decided to attempt to adapt the existing extraction procedures to Beale's modification of Zimmermann reaction. During this work it appeared that it was possible to use higher concentrations of KOH and m-dinitrobenzene and still obtain reagent blank values of zero in the range > 420 nm. At the same time the sensitivity improved. In addition purification of the methanol could be omitted and KOH solution used without filtration. With the concentration used in this method i.e. KOH 4.0 mol/l in the reaction mixture – it was necessary to reduce the incubation temperature from 37° C to 13–15° C in order to obtain a suitably long incubation time and to avoid destruction of the Zimmermann chromogens. 35 minutes were found to give the maximum colour development for androsterone and pooled dichloroethane extracts from the estimation of Tot. 17-KS and Tot. 17-OHCS, but 25–60 minutes produced no significant difference. It is important that the mixture of KOH and m-dinitrobenzene in methanol should not become warm at any point, as this can destroy Zimmermann chromogens. Hence the mixture is prepared and used during cooling to 0° C and the samples are cooled to 0° C after colour development.

With the optimal conditions thus obtained for the Zimmermann reaction, studies were then made on the different extraction systems. A choice of an extraction system on a rational chemical basis is difficult because the systems used are multi component systems, where the composition of the two phases obtained after equilibration is determined by the relative amounts of base water ethanol or methanol and the organic solvent. In addition the specific 17-ketosteroid reaction products to be extracted have a complicated composition. As mentioned above, colourless unionised and coloured quinoid forms with different solubility and stability are found. Finally the individual 17-ketosteroids are different with regard to polarity, e.g. 11-OH-AE is more polar than the 11-deoxy-17-ketosteroids, androsterone, aetiocholanolone and DHEA. The following experiments were conducted using dichloromethane as the organic solvent and had the object of developing an extraction system, which could extract the unionised colourless and stable reaction products of the four above mentioned 17-ketosteroids quantitatively.

Experiments

1. The distribution of unionised and quinoid forms of Zimmermann reaction products from androsterone and 11-OH-AE between the different phases, was studied by equilibration of systems containing 4 ml dichloromethane and different amounts of water. acetic acid, methanol and KOH. Phase equilibra-

238
tion and measurement of the extinction of the dichloromethane phase was carried out as described under method. The results are shown in Table 1. When there was no quinoid form in the water phase, the dichloromethane phase contained all the Zimmermann chromogens in their unionised forms; this has been confirmed by reextraction of the water phase. It can be seen that the concentration of KOH must be sufficiently low in order that the 17-ketosteroid reaction products can be extracted quantitatively in their unionised colourless forms. KOH concentrations of less than 0.16 mol/l gave quantitative extraction for both androsterone and 11-OH-AE. An acidified water phase gave a yellow dichloromethane extract with a relatively high blank value and was therefore not further studied. With a KOH concentration of 0.08 mol/l which was chosen for routine procedures, a water phase of 5 ml with 20 % (v/v) methanol was obtained. If the system contained more than approximately 30 % (v/v) methanol with the same KOH concentration, the methanol was distributed between both phases and the unionised forms of

**Table 1.**

Distribution of unionised and quinoid forms of Zimmermann reaction products from Androsterone and 11-OH-AE in two phase systems. 4 ml dichloromethane and volumes of water or 4 ml acetic acid of different concentrations were added to 1 ml reaction product with KOH 4 mol/l.

<table>
<thead>
<tr>
<th>KOH mol/l in water phase</th>
<th>Androsterone</th>
<th>11-OH-AE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>water phase</td>
<td>CH₂Cl₂ phase</td>
</tr>
<tr>
<td>1 ml water</td>
<td>quinoid</td>
<td>quinoid-unionised</td>
</tr>
<tr>
<td>2 ml water</td>
<td>quinoid</td>
<td>quinoid-unionised</td>
</tr>
<tr>
<td>3 ml water</td>
<td>1.00</td>
<td>unionised 97 %</td>
</tr>
<tr>
<td>4 ml water</td>
<td>0.80</td>
<td>unionised 99 %</td>
</tr>
<tr>
<td>4 ml CH₃COOH:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50 mol/l</td>
<td>0.40</td>
<td>unionised 102 %</td>
</tr>
<tr>
<td>0.70 mol/l</td>
<td>0.24</td>
<td>unionised 99 %</td>
</tr>
<tr>
<td>0.80 mol/l</td>
<td>0.16</td>
<td>unionised 99 %</td>
</tr>
<tr>
<td>0.85 mol/l</td>
<td>0.12</td>
<td>unionised 101 %</td>
</tr>
<tr>
<td>0.90 mol/l</td>
<td>0.08</td>
<td>unionised 98 %</td>
</tr>
<tr>
<td>0.95 mol/l</td>
<td>0.04</td>
<td>unionised 100 %</td>
</tr>
</tbody>
</table>

* Percentages indicate the amount of Zimmermann reaction products found on unionised form, the amount for KOH 0.04 mol/l in the water phase arbitrarily being set to 100 %.
11-OH-AE could not be extracted quantitatively. In addition a study of the absorption spectra for urinary 17-ketosteroids showed that the purest spectra were obtained with a pure dichloromethane phase.

2. The stability of Zimmermann chromogens during extraction was studied by the addition of dichloromethane after the preparation of the water phase. It was possible to note a decreased extinction after an interval of 30 seconds between the addition of acetic acid and dichloromethane. In the routine procedure it was therefore important that dichloromethane should be added first and thereafter acetic acid in simultaneous rotation.

The stability of the unionised chromogens in the colourless or slightly coloured extract was good as expected. No changes were noted on standing for 3 h at 0°C, and the extract is probably stable for a much longer period, as the test period only lasted three hours. The extract is also stable at room temperature, but owing to the low boiling point of dichloromethane it is more practical to store the samples from a larger series in a water bath at 0°C. The extracts may be cloudy, but actual emulsion formation does not occur, and comparison with centrifuged clear extracts gave exactly the same results.

3. The addition of methanol or ethanol to the colourless extract did not produce the quinoid colour, in agreement with the fact that the pure dichloromethane phase does not contain KOH, which is necessary for the transformation of the unionised to the quinoid form. Colour development with 0.6 ml KOH/methanol 60 mmol/l gave a fine reproducible colour which, protected from light, is stable for approximately 10 minutes at 0°C, but only for approximately 2 minutes at room temperature. Measurement must therefore be carried out immediately after the colour development, but this is easily done in practice, as all the samples from a large series can be stored for at least 3 hours, providing the colour developer has not been added. The addition of larger amounts of KOH as the colour developer does not produce increased colour intensity or stability, but can produce turbidity and a yellow reagent blank.

Reproducibility

The reproducibility of the Zimmermann reaction and the extraction procedure can be seen from the standard deviation for 30 values of $E_{530}^{\text{m}}$ for 30 μg androsterone. The mean value of the 30 measurements from a period of 6 months – including the use of reagents prepared at various times – was $E_{530}^{\text{m}} = 0.390$. The standard deviation was 0.012.

Linearity

Results in Table 2 show good linearity between the amount of 17-ketosteroid and the extinction for androsterone, and pooled dichloroethane extracts from the estimations of Tot. 17-KS and Tot. 17-OHCS.
Table 2.
Linearity between extinction and amount of 17-ketosteroids for Androsterone and pooled extracts from determinations of Tot. 17-KS and Tot. 17-OHCS.

<table>
<thead>
<tr>
<th>Androsterone µg</th>
<th>( \frac{E^530}{\mu g} )</th>
<th>Tot. 17-KS Extract ml</th>
<th>( \frac{E^530}{ml} )</th>
<th>Tot. 17-OHCS Extract ml</th>
<th>( \frac{E^530}{ml} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.012</td>
<td>1</td>
<td>0.063</td>
<td>1</td>
<td>0.124</td>
</tr>
<tr>
<td>3</td>
<td>0.011</td>
<td>2</td>
<td>0.071</td>
<td>2</td>
<td>0.116</td>
</tr>
<tr>
<td>6</td>
<td>0.011</td>
<td>4</td>
<td>0.069</td>
<td>3</td>
<td>0.114</td>
</tr>
<tr>
<td>12</td>
<td>0.0125</td>
<td>7</td>
<td>0.069</td>
<td>5</td>
<td>0.113</td>
</tr>
<tr>
<td>24</td>
<td>0.0123</td>
<td>9</td>
<td>0.068</td>
<td>7</td>
<td>0.113</td>
</tr>
<tr>
<td>30</td>
<td>0.0127</td>
<td>10</td>
<td>0.065</td>
<td>8</td>
<td>0.114</td>
</tr>
<tr>
<td>36</td>
<td>0.0125</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>0.0129</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.0128</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.0129</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Colour equivalents
The relative colour equivalents for some 17-ketosteroids and for dihydrotestosterone are given in Table 3. The most important 17-ketosteroids in relation to the methods described in this work are androsterone, aetiocholanalone, DHEA, and 11-OH-AE. Androsterone is used as the analytical standard but is converted to 11-OH-AE which is again converted to tetrahydrocortisol which is an expression of Tot. 17-OHCS. Similarly 11-oxy-17-OHCS is calculated as tetrahydrocortisol and 11-deoxy-17-OHCS as pregnanetriol, as the androsterone standard in this case is converted to aetiocholanalone.

By comparing the colour equivalents for 11-keto-aetiocholanalone, androstenedione, and androstenetrione it can be seen that the oxo groups in other

Table 3.
Relative weight colour equivalents of extracted Zimmermann chromogens calculated from \( E^530 \) (arb. value).

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Androsterone</td>
<td>100*</td>
</tr>
<tr>
<td>Aetiocholanalone</td>
<td>111</td>
</tr>
<tr>
<td>11-OH-aetiocholanalone</td>
<td>96</td>
</tr>
<tr>
<td>Dehydrocypandosterone</td>
<td>88</td>
</tr>
<tr>
<td>11-keto-aetiocholanalone</td>
<td>77</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>22</td>
</tr>
<tr>
<td>Androstenetrione</td>
<td>5</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>4</td>
</tr>
</tbody>
</table>
positions in addition to C-17, result in lower colour equivalents, particularly for the last two mentioned 17-ketosteroids. The cause of this is not less chromogenicity in the Zimmermann reaction but that these compounds partly remain in the water phase during the extraction. This cannot have an appreciable effect on the Tot. 17-KS values as the urine contains only very little androstenedione and androstenetrione. Dihydrotestosterone, a 3-mono-ketosteroid has normally 20–30% of the chromogenicity of androstosterone. During extraction the colour equivalent is reduced to 4% of that for androsterone. The same probably applies to the other 3-mono-ketosteroids and this is desirable as these compounds belong to the unspecific chromogens.

**Specificity**

In order to evaluate the specificity of the extraction procedure, a comparison was made between the spectra for extracted Zimmermann chromogens and chromogens from Zimmermann reaction without extraction, for 11 urine extracts from Tot. 17-KS estimations and 8 urine extracts from Tot. 17-OHCS estimations. Some of the spectra are shown in Fig. 1. The extracted chromogens

\[\text{Absorption spectra obtained for assays carried out with and without extraction of the specific Zimmermann chromogen from estimations of Tot. 17-KS and Tot. 17-OHCS.}\]

\[\text{The spectra recorded with a Spectronic 505 spectrophotometer.}\]

show a similarity to androsterone. In Table 4 the calculated uncorrected values for non extracted and extracted chromogens are shown. The difference is considerable for all the urines. Allen corrected values are also given as a numerical evaluation of the similarity of the spectra for extracted chromogens and androsterone. It can be seen that the uncorrected and corrected values for extracted chromogens are almost identical. Correction by reading on several wave lengths are thus unnecessary.

Another evaluation of the specificity was carried out by comparing $E_{530}$ values for Tot. 17-OHCS with the sum of the $E_{530}$ values for the corresponding 11-deoxy and 11-oxy fractions. The value of $F = \frac{(E_{11-deoxy} + E_{11-oxy}) \cdot 100}{E_{Tot. 17-OHCS}}$ must be approximately 100% providing the partition chromatography does not remove larger amounts of unspecific chromogens than those eliminated by the dichloromethane extraction. The fact that chromatography can remove considerable amounts of unspecific chromogens was clearly demonstrated, as the magnitude of $F$ at the beginning of this investigation, where extraction of the Zimmermann chromogens was not performed, varied between 40 and 92% with an average of 70% for 53 urines. After the commencement of the

<table>
<thead>
<tr>
<th>Urine</th>
<th>Tot. 17-KS mg/l</th>
<th>Tot. 17-OHCS mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unextracted</td>
<td>extracted</td>
</tr>
<tr>
<td>581</td>
<td>24.2</td>
<td>19.1</td>
</tr>
<tr>
<td>595</td>
<td>16.6</td>
<td>5.2</td>
</tr>
<tr>
<td>596</td>
<td>9.8</td>
<td>6.5</td>
</tr>
<tr>
<td>599</td>
<td>17.2</td>
<td>10.3</td>
</tr>
<tr>
<td>639</td>
<td>6.3</td>
<td>3.0</td>
</tr>
<tr>
<td>640</td>
<td>3.7</td>
<td>2.3</td>
</tr>
<tr>
<td>641</td>
<td>2.7</td>
<td>1.4</td>
</tr>
<tr>
<td>642</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>647</td>
<td>6.3</td>
<td>4.4</td>
</tr>
<tr>
<td>654</td>
<td>7.1</td>
<td>3.1</td>
</tr>
<tr>
<td>656</td>
<td>15.8</td>
<td>9.5</td>
</tr>
<tr>
<td>664</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Table 4.

Values for unextracted and extracted Zimmermann chromogens from the same urine extracts produced during the estimation of Tot. 17-KS and Tot. 17-OHCS. $E_{530}$ was used for the calculation of uncorrected values and $E_{corr.}^{530} = E_{450}^{530} \cdot \frac{E_{450}^{530} + E_{610}^{530}}{2}$ for the Allen corrected values. Tot. 17-KS was calculated as Androsterone and Tot. 17-OHCS as 11-OH-AE.
extraction method fractionation of 196 normal urines gave F values of 83–105% with an average of 92%. Similarly by fractionation of a pooled extract from determinations of Tot. 17-KS F was 51% without and 91% with the extraction method. These results thus show that dichloromethane extraction approximately eliminates the same amount of unspecific chromogens as partition chromatography.

Reliability of the Methods

The specificity has been considered in a previous paragraph.

Precision. The precision of the methods for Tot. 17-KS and Tot. 17-OHCS was evaluated by the standard deviations at 2 excretion levels. The values used for this were chosen at random from 196 estimations of normal urines. The reproducibility of the fractionating procedure was evaluated by the standard deviations for the two fractions of a pooled extract with a relatively high 11-deoxy content. The results are shown in Table 5.

Accuracy. Representative steroid conjugates with the exception of DHEA sulphate were not available. Exact recovery experiments could not therefore be carried out.

It is well-known that treatment with hot acid during the estimation of Tot. 17-KS can partly convert and/or destroy some 17-ketosteroids. In a comment to the M. R. C. (1963) standard method for the estimation of Tot. 17-KS, Metcalf (1964) states that 50% of DHEA calculated as Zimmermann chromogen is destroyed by the acid treatment. An acid hydrolysis loss of approximately

<table>
<thead>
<tr>
<th></th>
<th>Range mg/l</th>
<th>Mean mg/l</th>
<th>Standard dev. mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tot. 17-KS</td>
<td>2.4–5.0</td>
<td>3.5</td>
<td>0.23</td>
</tr>
<tr>
<td>(30 duplicates)</td>
<td>5.5–20.0</td>
<td>10.0</td>
<td>0.36</td>
</tr>
<tr>
<td>Tot. 17-OHCS</td>
<td>4.1–8.4</td>
<td>6.7</td>
<td>0.25</td>
</tr>
<tr>
<td>(30 duplicates)</td>
<td>9.3–16.9</td>
<td>12.0</td>
<td>0.38</td>
</tr>
<tr>
<td>11-deoxy-17-OHCS</td>
<td>3.9</td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>(21 estimations of a pooled extract)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-oxy-17-OHCS</td>
<td>9.0</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>(21 estimations of a pooled extract)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
20% is found with the technique used here, when pure DHEA or DHEA sulphate in water is subjected to acid hydrolysis. On the other hand, much less can be recovered from urine. Table 6 shows some of the results of the recovery experiments performed on urines to which different 17-ketosteroids were added before acid hydrolysis. It can be seen that androsterone, aetiocholanolone, and 11-OH-AE are stable with regard to chromogenicity in the Zimmermann reaction. On the contrary DHEA is unstable and by comparison with the above mentioned 80% recovery of DHEA or DHEA sulphate from water, it can be seen that the recovery per cent from urine is lower, and lowest and more variable for the free DHEA than for DHEA sulphate. In recovery experiments using DHEA added to urines diluted 0, 1/2, 1/5 and ∞ (water) a recovery per cent was found in agreement with the above of 47 – 55 – 67 – and 80%. The acid effect alone thus cannot be the cause of the relatively low and varying recovery per cents for urine to which DHEA or DHEA sulphate had been added. Beher & Gaebler (1951) state that bile acids and proteins in the urine in variable quantities can effect the recovery of DHEA. Similarly Vestergaard (1962) found that certain urines can show various degrees of a «quenching» effect on DHEA recovery. These problems have not been the subject of a more thorough study in the present investigation. The DHEA content of urine both normally and in the majority of pathological conditions, constitutes 10–20% of Tot. 17-KS and in rare cases, where the DHEA is increased, a fractional analysis of the 17-ketosteroids will be required in any case.

Recovery experiments with Tot. 17-OHCS estimation were carried out with tetrahydrocortisol, and the recovery on the addition to urine was 71–76% (mean 74%) for 10 urines compared with 86–92% (mean 88%) from water after 10 estimations.

The reliability of the column chromatography was controlled by repeated

<table>
<thead>
<tr>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aetiocholanolone (1)</td>
</tr>
<tr>
<td>11-OH-AE (1)</td>
</tr>
<tr>
<td>Androsterone (10)</td>
</tr>
<tr>
<td>DHEA sulphate (4)</td>
</tr>
<tr>
<td>DHEA (6)</td>
</tr>
</tbody>
</table>

Table 6.
Recovery of pure 17-ketosteroids added to urines (20 mg/l) prior to acid hydrolysis.
routine fractionations of aetiocholanolone and 11-OH-AE. The recovery for the two compounds was always between 94 and 100%.

A normal material for the methods will be published at a later date.

ACKNOWLEDGEMENTS

The author wishes to thank Mrs. G. Behncke, Mrs. M. Nielsen and Mrs. A. Nielsen for their interest and valuable assistance during the investigation.

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