A SIMPLIFIED METHOD FOR THE DETERMINATION OF NEUTRAL 17-KETOSTEROIDS IN SMALL VOLUMES OF URINE

By E. Birket-Smith

The comprehensive literature on methods for the determination of neutral 17-ketosteroids (17-KS) in urine gives the impression – as also emphasized by Kassenaar et al. (1950) – that practically every laboratory performing this analysis employs its own particular technique. This might be taken to indicate that none of the present methods is perfectly satisfactory. Hence it seemed desirable to investigate this problem again, with a special view to the possibility of further simplification of the technique.

The starting-point for the studies reported in the following has been the «micromethod» published by Hamburger & Rasch (1948), which naturally has been used as a reference method. This seems justifiable as the normal values obtained with this method – according to the material gathered by Hamburger (1948) – agree with the findings reported by most other investigators. From the following discussion, however, it will be evident that the results hardly represent the absolute 17-KS-values.

A schematic presentation of the various steps in the analysis with the technique of Hamburger & Rasch and with the modification described here is given in Table 1. From this it will be noticed that the procedure naturally falls in two parts: 1) production of a urinary extract with the highest possible concentration of 17-KS in proportion to the concentration of interfering substances, and 2) determination of the 17-KS content of this extract by colorimetry.

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The pure steroid preparations used have been generously supplied by Ciba Limited, Basel.
While the most previous methods were based on extraction of an amount of urine corresponding to \(\frac{1}{4}-\frac{1}{2}\) of the 24-hour urine. Hamburger & Rasch reduced the volume of urine extracted to \(\frac{1}{50}\) of the 24-hour output employing only a \(\frac{1}{4}\) of the extract obtained for the final colour reaction.

It might seem most satisfactory to employ a fixed fraction of the urine for the analysis, but when the object is to estimate the excretion within a period shorter than 24 hours this has proved inconvenient, because of the great variations in the urinary output. I have therefore chosen to extract a constant volume of urine and have, after careful consideration, decided on 10 ml. By the use of a constant amount of urine, it was theoretically to be expected that a greater number of the analytical results than has previously been found would fall

Table 1.

Schematic presentation of the various steps in the analytical procedure according to Hamburger & Rasch (A) and in the modification described (B).

A.

1/50 of the 24-hour urine + 1/10 vol. 40\% sulphuric acid.
Boiling for 25 minutes.
Cooling.
Transference to separating funnel by two washes of distilled water.
Extraction with about 40 ml. ether.
Extract washed once with saturated solution of sodium bicarbonate, twice with 2 n sodium hydroxide and twice with distilled water.
Drying with anhydrous sodium sulphate.
Filtration.
Evaporation of ether.

Residue is dissolved in 0.8 ml. absolute alcohol.
Zimmermann reaction is performed with 0.2 ml. of this solution in duplicate:
0.2 ml. of the solution
+ 0.2 ml. 2\% m-dinitrobenzene
+ 0.2 ml. 2.5 n potassium hydroxide
Incubation for 60 min. at 25° in the dark.
Dilution with 9.4 ml. absolute alcohol.
Reading of the absorption at wave lengths 520 and 470 mμ.

B.

10 ml. urine + 1 ml. 40\% sulphuric acid.
Boiling for 25 minutes.
Cooling.
Transference to separating funnel by two washes of distilled water.
Extraction with about 25 ml. ether.
Filtration of extract through layer of powdered sodium hydroxide.

Evaporation of ether.

Residue is dissolved in 0.2 ml. absolute alcohol + 0.2 ml. m-dinitrobenzene - if required under slight heating.
0.2 ml. 2.5 n sodium hydroxide in absolute alcohol is added.

Incubation for 30 min. at 37° in the dark.
Dilution with 9.4 ml. absolute alcohol.
Reading of the absorption at wave lengths 520 and 470 mμ.
outside the optimal range of measurements. In practice, however, this has proved of no significance.

**PREPARATION OF THE URINARY EXTRACT**

**A. Hydrolysis.** The water-soluble 17-KS conjugates present in the urine have to be split into fat-soluble free steroids prior to the extraction, but a sufficiently strong hydrolysis brings about a partial destruction of the steroids at the same time (Bitman & Cohen, 1949. Butenandt & Dannenbaum, 1934, Dingemanse & Laqueur, 1938. Friedgood et al., 1943. Gallagher, 1944, Wolfe et al., 1941). Up to the present no suitable solution of this paradoxical problem has been found. Thus simultaneous hydrolysis and extraction (Hamburger & Rasch, 1948), hydrolysis at temperatures below boiling point (Drekter et al., 1947, Kassenaar et al., 1950) and enzymatic hydrolysis appear to offer no advantage, and by comparison of seven different procedures Jensen & Tötterman (1952 a) found signs of steroid destruction, particularly of dehydroisoandrosterone, even in the two methods giving the highest yields. Some »differential« or »fractional« hydrolytic procedures seem to present a possibility for reducing the destruction (Dobriner & Lieberman, 1950, Jensen & Tötterman, 1952 b) but have been found too complicated for routine assessment of the total 17-KS-output.

In consideration of the above I have retained the usual hydrolysis by boiling with acid. In spite of the reduction in the duration of the hydrolysis obtainable by using concentrated hydrochloric acid, I have used \(\frac{1}{10}\) vol. of 40% sulphuric acid as indicated by Hamburger & Rasch, because of the inconveniences involved in handling concentrated hydrochloric acid.

As a close standardization of each step in the procedure appears essential owing to the steroid destruction which presumably occurs, I have found it advisable to reproduce the experiments already reported on the optimal duration of the hydrolysis. In keeping with the previous investigators, I have found the greatest yield of 17-KS after hydrolysis on an electrical heating plate for 25 minutes. The hydrolysis is timed from the moment when all the specimens on the heating plate have started boiling, for which reason among others it is an advantage to work with a constant volume of urine.

**B. Extraction.** After quantitative transfer of the hydrolyzed urine to a separating funnel by two washes of distilled water the fat-soluble substances are extracted by vigorous shaking for 1 min. with about 25 ml. ether. In a not inconsiderable number of cases this gives rise to emulsification of the aqueous phase in the ether, rendering any further analysis impossible.

After having observed that the emulsion formed can in most cases be broken by shaking with strong alkali, I have tried to avoid this emulsification by adding 2 ml. of 10 n sodium hydroxide prior to the extraction. In this way, emulsification has become considerably less frequent.
Comparison between results obtained by purification of ether extracts with NaOH pellets and ad modum Hamburger & Rasch. 8 extracts with each method, all from the same batch of urine, double Zimmermann reaction performed on each extract.

### Table 2.

<table>
<thead>
<tr>
<th></th>
<th>A) «Pellet method»</th>
<th>B) Hamburger &amp; Rasch</th>
</tr>
</thead>
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<tr>
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<td>D&lt;sub&gt;470&lt;/sub&gt;</td>
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<tr>
<td>8.</td>
<td>163</td>
<td>144</td>
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</table>

\[ m_A = 18 \pm 1.9 \quad \quad \quad \quad m_B = 33 \pm 0.7 \]

\[ t = 7.5 \]

Condition of significance corresponding to the 1% level: \( t > 2.977 \).

Mean value of the quotient \( D_{470} / D_{320} \):

A) \( 1.00 \)

B) \( 0.91 \)

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**C. Purification of the ether extract.** A purification of the ether extract is necessary in order to free it from interfering chromogens. The commonly adopted procedure consists in several successive shakings with alkali and water, followed by drying for removal of the water combining with the ether (see Table 1), but this method is rather time-consuming.

Recently, Drekter et al. (1950) have reported that the entire procedure may be limited to a single shaking with sodium hydroxide pellets. On examination of a number of urine specimens with purification after the usual procedure as well as after this «pellet method», however, the latter was found to show a
Table 3.
Comparison between results obtained by purification of ether extracts by filtration through powdered NaOH and ad modum Hamburger & Rasch. Several analyses with each method on each of three different batches of urine.

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<tr>
<th>Urine</th>
<th>D_{520}</th>
<th>D_{470}</th>
<th>µg.</th>
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\[
\frac{D_{470}}{D_{520}} = 1.03. \quad m_A = 29 \pm 1.6.
\]

\[
t = 1.08. \quad \text{Condition of significance (1% level): } t > 2.977.
\]

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\[
\frac{D_{470}}{D_{520}} = 0.78. \quad m_A = 99 \pm 0.9.
\]

\[
t = 2.69. \quad \text{Condition of significance (1% level): } t > 2.977.
\]

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\[
\frac{D_{470}}{D_{520}} = 0.84. \quad m_A = 74 \pm 1.6.
\]

\[
t = 2.00. \quad \text{Condition of significance (1% level): } t > 3.169.
\]
distinct tendency to give lower yields. This impression has been further confirmed by the results from 8 analyses after both methods performed on the same batch of urine, since they showed significantly lower values for the »pellet method« than for the usual procedure (Table 2).

The lower results obtained with the »pellet method« might mean a better purification of the extract and so give a more correct expression for the actual 17-KS content. That this is not the case appears from the observation that the average quotient of D_{470}/D_{520} in the final colour reaction, the value of which gives a fair indication of the contamination with nonspecific chromogens, is higher after purification with the pellets than with the ordinary method. In consequence, the lower yields obtained with the »pellet method« must be due to other factors, so far uncontrollable, and hence this method has been considered less suitable. It should be mentioned, however, that Vestergaard (1951) claims to have obtained satisfactory results with the pellet method.

Further experiments have shown that a satisfactory purification may be obtained by filtration of the crude ether extract through a layer of powdered sodium hydroxide placed in a piece of filter paper. The amount of sodium hydroxide is apparently of no particular significance (about 10 gm. is suitable) but care has to be taken that the entire extract actually passes through the sodium hydroxide layer. The separating funnel is washed three times with about 2 ml. ether, which is also poured through the filter. ensuring that traces of 17-KS retained are included in the filtrate.

The results of repeated analyses carried out with this method as well as with the ordinary procedure on 3 different batches of urine are recorded in Table 3. To exclude any possible variations due to the hydrolysis all the portions of the same batch have been hydrolyzed as a whole.

From Table 3 it will be seen that the mean values for each batch of urine do not differ significantly from each other. Furthermore the difference between the means goes in the opposite directions in the various experiments. It seems therefore justifiable to assume that no systematic difference exists between the results obtained with the two different methods.

This conclusion has been further confirmed by the results of single analyses with the modified method to its full extent as compared with the results of the reference method applied to a large number of different urine specimens (Fig. 3).

Evidence of the adequacy of the modified purification procedure is as mentioned above found in the mean values for D_{470}/D_{520}. In no instance is the difference between the values for this quotient obtained with the two methods significant. Taken together, however, they would seem to indicate a slightly less efficient purification with the sodium hydroxide method, but the difference is too slight to be of any practical significance.

Owing to its water-absorbent character, storage of the powdered sodium
hydroxide presented a particular problem. Still, this difficulty appears to have been overcome satisfactorily by storing the sodium hydroxide in ether in a tightly stoppered bottle, as it has then always proved capable of taking up any trace of water in the extracts.

D. Evaporation of the ether. This was carried out under slightly reduced pressure from a suction pump with an infra-red lamp as the source of heat to prevent danger of explosion. Care should be taken to remove the tubes from the heat immediately after complete evaporation of the ether as it may be difficult to obtain a quantitative solution of the residue if this has been overheated.

DETERMINATION OF THE 17-KS CONTENT OF THE EXTRACT

The 17-KS content of the dry residue after evaporation of the ether can be determined in three fundamentally different ways. The principle most widely adopted, however, is undoubtedly the colour reaction of Zimmermann (1935, 1936) with meta-dinitrobenzene and potassium hydroxide, as the reaction worked out by Pincus (1943) with antimony trichloride allows of the determination of the beta-17 KS only, and the polarimetric method described by Barnett et al. (1946) and Butt (1950) requires a complicated technique.

The Zimmermann reaction is not absolutely specific for 17-KS as it is merely a reaction for the presence of ketone groups. Still, when using sufficiently purified extracts and reading the colour intensity at the absorption maximum characteristic of 17-KS, this reaction gives results of practical value, especially when using a correction for the presence of nonspecific chromogens based on the different absorptive properties of these compounds (Fraser et al., 1941, Talbot et al., 1942).

The Zimmermann reaction is in general employed in one of the two modifications indicated by Callow et al. (1938) and Holtorff & Koch (1940), respectively. Technically the latter is a little simpler, but it does not allow of the correction for nonspecific chromogens, for which reason I have chosen to work with the first-mentioned modification, in spite of the drawback due to the lesser stability of the reagents employed.

E. Reaction mixture. The reagents employed for Callow's modification of the Zimmermann reaction comprise a 2\%/ (w/v) solution of meta-dinitrobenzene in absolute alcohol, and a 2.5 n solution of potassium hydroxide, also in absolute alcohol. These solutions are relatively unstable. Still, the m-dinitrobenzene solution has proved to keep for at least two weeks when stored in a tightly stoppered dark bottle. It has to be kept at room temperature as crystallization takes place at lower temperatures. The potassium hydroxide solution
has proved stable for at least one week when it is stored in a tightly stoppered bottle at a temperature of 15° below zero or colder. The stability of the reagents is secured by including a standard sample of 100 μg. dehydroisoandrosterone in the analyses every day.

In the »micromethod« of Hamburger & Rasch the residue after the evaporation is dissolved in 0.8 ml. absolute alcohol, after which 0.2 ml. of the solution is transferred to a test tube for further analysis. This makes it possible to perform the reactions in duplicate but also introduces an important source of error. Thus quantitative solution of the residue is often so difficult as to necessitate a brief heating, but even relatively slight evaporation of alcohol before the sample is taken out for the colour reaction will mean a not inconsiderable error in the analytical result.

This source of error is avoided by performing the colour reaction on the total residue in the very tube in which the evaporation took place. The modified procedure is as follows: the residue is dissolved in 0.2 ml. absolute alcohol + 0.2 ml. m-dinitrobenzene solution – if required under light heating, as evaporation here plays a minor role since the mixture has to be considerably diluted before the reading. Finally, 0.2 ml. potassium hydroxide solution is added and the reagents are mixed thoroughly.

**F. Incubation.** Usually the reaction mixture is incubated at a temperature of 25°, but the employment of this temperature appears to be a convention rather than founded on experimental studies. As, furthermore, this temperature for various reasons was found inconvenient, it was decided to look further into the question of the dependence of the colour reaction on the temperature and time.

Originally Zimmermann (1935) let the reaction mixtures stand at room temperature for one hour. In a subsequent study he investigated the susceptibility of the reaction to changes in temperature and found agreement with van't Hoff's law as a rise in temperature from 15° to 25° brought about an increase in the rate of the reaction »und damit der Farbenintensität« to 2.2 times the original value. He also states that the shape of the absorption curve is not affected by the temperature. The length of the incubation period is stated to have been chosen arbitrarily, because »man kann nicht im Maximum ablesen, da durch die starke Hydroxylionenkonzentration vorher bereits Zersetzung eintritt, so dass das Beersche Gesetz ungültig wird«.

Callow et al. (1938) employed incubation at 25° for 1 hour, as they found a typical maximum curve with a maximum at 60 min. for the variation of absorption with time. They found further an increase in the absorption amounting to 3% on increasing the temperature by 1°.

In their modification, Holtorff & Koch (1940) incubates the mixture at 25° for 45 min. »because the reaction is almost complete« after this period. In contrast, Engström & Mason (1943) claim that by this very method it takes 75 min.
to obtain maximal absorption, whereas it takes only 15 min. with the modification of Callow et al.

More thorough investigations on the development of the colour intensity with the time have been reported by Nathanson & Wilson (1943) for four different modifications of the Zimmermann reaction. The reactions were performed at a temperature of 25°, and the authors then found that the Callow modification gives a maximal colour intensity within 80–100 min. and 35–45 min. for pure androsterone and urinary extracts, respectively, in both instances followed by a slow decrease. Similar plateau-shaped curves for the development of the colour intensity have been obtained by Cahen & Salter (1944) employing the modification of Holtorff & Koch. Finally, it should be mentioned that Pearson & Giaccone (1948) with a technique somewhat differing from the current methods read the results after only 20 min. at 25°.

![Figure 1](https://example.com/figure1.png)

Fig. 1.

Variation of the absorption (expressed by the D-values) at wave lengths 520 and 470 m\(\mu\) with the length of incubation at 25° and 37°, respectively, for pure dehydroisoandrosterone.
The present studies have primarily been aimed at ascertaining the variation of the colour intensity expressed by the D-values (D = density) at wave lengths of 520 and 470 m\(\mu\) with the time at the temperatures of 25\(^\circ\) and 37\(^\circ\) for pure dehydroisoandrosterone (Fig. 1).

In agreement with previous investigations, these experiments resulted in typical plateau-shaped curves at both wave lengths. On comparison of the curves obtained at 25\(^\circ\) and at 37\(^\circ\) it is evident that the maximal colour intensity noted does not depend on the temperature. On the other hand, the maximal absorption at 37\(^\circ\) is already obtained after 20 min. as against 40 min. at 25\(^\circ\). Furthermore, these curves demonstrate that the quotient of \(D_{470}/D_{520}\) is independent of the temperature as far as the plateau-part of the curves is concerned: after 60 min. at 25\(^\circ\) this quotient is 0.63, and after 30 min. at 37\(^\circ\) it is 0.64.

After this the absorption curves were determined for pure dehydroisoandrosterone and for various urinary extracts after incubation at 25\(^\circ\) for 60 min., and at 37\(^\circ\) for 30 min. (Fig. 2). From Fig. 2 it is evident that the curves are practically identical.

In addition, the absorption curves have been established for 3 different «non-ketonic fractions» prepared by fractionation with Girard's «reagent T» in the

![Absorption curves for 0.10 mg. dehydroisoandrosterone (DHA) and for a urinary extract (U) after incubation at 25\(^\circ\) for 60 minutes and at 37\(^\circ\) for 30 minutes.](image-url)
modification given by Sprechler (1950). Again, the curves were found to be identical for the two modifications of the incubation. In order to ensure against interference by $\Delta_1$-3-ketosteroids the absorption curve for pure testosterone was determined. As the curve obtained resembles rather closely the curves for the non-ketonic fractions, such interference seems to be excluded.

Finally, with regard to the calculation of the correction for nonspecific chromogens, the quotient $D_{470}/D_{520}$ was determined for 10 non-ketonic fractions after reaction at $37^\circ$ for 30 min. The mean value thus obtained $- 1.269$ - corresponds quite closely to the value given by Hamburger & Rasch for the reaction after 60 min. at $25^\circ$: 1.270.

In consequence it was only natural to expect that 17-KS determinations on urinary extracts would give identical results without regard to the modification of the incubation procedure employed. Indeed, this assumption has been fully confirmed by determination of the 17-KS content of 47 different urine specimens after both procedures, as no systematic difference between the results could be demonstrated. It was therefore decided to perform the colour reaction with the modification here described.

G. Reading of the colour reaction has been performed in a Beckman spectrophotometer (model DU) at the wave lengths 520 and 470 m$\mu$ compared with a blank consisting of the reagents $+ 0.2$ ml. absolute alcohol, after dilution of the tests as well as of the blank to 10 ml. with absolute alcohol. The reading must take place within 20 minutes after the dilution as the colour fades slowly.

The 17-KS content of the samples is calculated from the D-values read very easily by means of a correction monogram as indicated by Hamburger (1948). The 17-KS content of the total portion of urine is then easily calculated.

REPRODUCIBILITY OF THE METHOD, ETC.

In order further to ensure against systematic deviations between results obtained with the method of Hamburger & Rasch and with the modified method here described, in addition to the preceding comparison of the two methods step by step, a number of urines have been analyzed with both methods to their full extent. The results of such double analyses on 30 different urine specimens are given in the correlation diagram presented in Fig. 3. From this it is evident that there is no systematic divergence between results obtained with the two methods. In conclusion, the results of these methods may be considered directly comparable.

In order to estimate the reproducibility of the modified method serial analyses on different batches of urine have been performed. As the standard deviation was found to be proportional to the absolute 17-KS content of the samples, it seems most expedient to put it in per cent of the mean value. In all
Diagram of the correlation between the results of analyses of 30 different urine specimens with the original method of Hamburger & Rasch (A) and with the modified procedure (B).

Table 4.
Serial analyses on different batches of urine for estimation of the dispersion of the results obtained with the modified method. Only the two series giving the highest and the lowest value for the standard deviation are recorded.

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<th>I</th>
<th>µg.</th>
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<tr>
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\[
\begin{align*}
\text{I} & \quad m_1 = 8.7 \\
\text{II} & \quad m_1 = 3.0 \\
\text{I} & \quad s_1 = \pm 0.52 = \pm 6.0\% \\
\text{II} & \quad s_2 = \pm 0.08 = \pm 2.7\%
\end{align*}
\]

the series the standard deviation has fallen within the extreme limits of about $\pm 3\%$ and $\pm 6\%$ (compare Table 4). For the sake of comparison it may be mentioned that in serial analyses according to the method of Hamburger & Rasch under otherwise uniform conditions, the standard deviation in the dif-
ferent series was found to vary between ± 7% and ± 14%. Hence, with regard to the reproducibility of the results, the modified method appears to be somewhat superior to the reference method.

The conclusion must be that the modified method here described is equal in every respect to or somewhat superior to the method of Hamburger & Rasch. Of practical importance, however, is the fact that the modified method represents a not inconsiderable simplification of the procedure and thus a saving of labour, as can be seen from Fig. 1. The consequence of this has been that the same person using the new modification of the method has been able to perform on an average twice as many analyses a day as previously.

**SUMMARY**

A simplified method for the determination of neutral 17-ketosteroids in urine based upon the »micro-method« of Hamburger & Rasch is described.

The main points in this modification are as follows:

1. A constant volume of urine – 10 ml. – is extracted instead of a fixed fraction of the 24-hour output.
2. 10 n sodium hydroxide solution is added to the hydrolyzed urine before extraction to counteract the tendency to emulsification.
3. Purification and drying of the ether extract is obtained simultaneously by filtration through powdered sodium hydroxide.
4. The Zimmermann reaction is performed on the entire dry extract in the same tube as the one in which the evaporation took place.
5. For the Zimmermann reaction the mixture is incubated at the more convenient temperature of 37° for 30 minutes instead of at 25° for 60 minutes.

On comparing the results obtained by the modified method and the original one it is evident that they are directly comparable, that the modified method with regard to the reproducibility of the results is somewhat superior to the original method and that it saves a not inconsiderable amount of labour and time.

**REFERENCES**

Vestergaard, P.: Acta endocrinol. 8, 193, 1951.