Elimination of Interfering Chromogens in the Zimmerman Reaction for Measuring 17-Ketosteroids

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When alcoholic potassium hydroxide and m-dinitrobenzene are used in the Zimmerman reaction (1) for the determination of 17-ketosteroids (17-KS), a purple complex is formed which has a broad absorption band in the green portion of the visible spectrum. Application of this reaction to urine extracts also gives rise to other chromogens which are brown in color. On occasion, the ratio of brown chromogens to Zimmerman color is so high that quantitation of 17-KS becomes difficult.

A variety of procedures have been proposed for eliminating the interference of these brown chromogens in quantitation of 17-KS. The method of Talbot et al. (2) uses mathematical correction after reading the absorption in both the green and blue areas of the visible spectrum. The accuracy of this method has been improved considerably by the use of Allen’s correction (3). Absorbance readings are made at the absorption maximum of the ketosteroid complex and at appropriate wave lengths equidistant on either side. By assuming linear absorption of interfering chromogens at the 3 points, it is possible to make a simple correction. This method is promising but...
somewhat time-consuming, and it necessitates the use of a spectrophotometer with good resolution.

Separation of the 17-KS by Girard's T reagent (4) has been accomplished, but the method is time-consuming. A practical method for preparing crude extracts was reported by Drekter et al. (5) in 1952, using ethylene chloride extraction followed by shaking with sodium hydroxide pellets. Treatment with sodium hydroxide pellets is reported to reduce the interfering chromogens sufficiently so that accurate measurements of the Zimmerman color can be made at one wavelength. We have found this to be true with some urine specimens, but others yield misleading results unless photometric correction is applied.

Attention has also been focused on purification of the Zimmerman complexes. Cahen and Salter (6) observed that partitioning between 60% ethanol and chloroform separated the 17-KS complex from the brown chromogens. Unfortunately, the separation is not complete, so that this method is not too acceptable. More recently, others have proposed the use of ether (7) or amylacetate (8) instead of chloroform. Kellie and Smith (9) have reported successful separation of individual 17-KS Zimmerman complexes by paper chromatography.

A number of years ago, investigation of Cahen and Salter's method by one of us (J.A.D.) led to the development of an improved procedure which has subsequently been used routinely for analysis of 17-KS and 17-ketogenic (17-KGS) steroids. Separation of the Zimmerman complex is accomplished by partitioning in the test tube used for the reaction. The purple 17-KS complex goes readily into the lower phase and permits quantitation in a filter photometer without transfer. The results appear to be of sufficient precision and accuracy to justify the following report.

METHODS
PREPARATION OF CRUDE EXTRACTS

The method of Drekter et al. (5) using ethylene chloride and sodium hydroxide pellets has been used for preparation of neutral 17-KS extracts from urine. For 17-KGS, the urine is first treated with sodium bismuthate according to the procedure of Norymberski (10) as modified by Sobel et al. (11). A portion of the ethylene chloride extract equivalent to 0.5-2.0 ml. of urine is evaporated to dryness in a photometer tube at 60° with a stream of nitrogen. The
Zimmerman reaction, as well as the purification of reagents, is carried out by Sobel's procedure (11), with minor modifications.

ZIMMERMAN REACTION AND SEPARATION OF INTERFERING CHROMOGENS

A saturated solution of aqueous KOH (approximately 12 N) is prepared and kept in a rubber-stoppered pyrex bottle at room temperature. This solution is suitable for use for at least 2 months. A portion of the KOH solution is diluted with ethanol just before use so that its concentration is 2.5 N. A 2% alcoholic solution of m-dinitrobenzene is prepared just before use. With each set of unknowns dehydroepiandrosterone (DHEA) standards (25 and 50 μg, in alcohol) are dried as above and included along with appropriate blanks. 0.2 ml. of m-dinitrobenzene and of alcoholic KOH solutions are added in that order to each tube, followed by immediate shaking. Thorough solution and mixing of the reagents and dried extract is essential. The mixture is allowed to stand in the dark for approximately 30 minutes at 25° ± 1. Quantitation of 17-KS is carried out immediately by one of the following procedures.

DEMETRIOU EXTRACTION PROCEDURE (METHOD D)

Six ml. of 40% propanol¹ in water and 3 ml. of methylene chloride are added in that order. The tubes are shaken vigorously to insure mixing of contents within 1 to 2 minutes after addition of the methylene chloride and centrifuged for a few minutes at approximately 2000 rpm. The methylene chloride-propanol phase (lower layer) increases in volume to approximately 5 ml. The purple steroid complexes are extracted into the lower layer, leaving the brown chromogens in the upper aqueous layer. The lower layer is optically clear and the absorbance may be quantitated in a filter photometer. In these studies a Klett-Summerson photometer with a No. 54 filter was used. No emulsions were encountered, but on rare occasions a second shaking and centrifugation was necessary to get good separation of the two layers.

PROCEDURE FOR APPLICATION OF ALLEN CORRECTION (METHOD A)

After the Zimmerman reaction was completed, dilution was made with 5 ml. of 70% ethanol and readings were carried out immediately at 440, 520 and 600 mμ in a Beckman DU spectrophotometer. Allen's correction formula (3) was applied to the data.

¹n-propanol from Matheson, Coleman, and Bell has given excellent results. Other manufacturer's products have occasionally resulted in turbidity.
RESULTS

SEPARATION OF INTERFERING CHROMOGENS

Aliquots of ethylene chloride extracts from urines were dried as above so that the residue was equivalent to 2 ml. of urine. The Zimmermann reaction and separation of interfering chromogens was carried out as described, including a 50 μg DHEA standard. Absorption curves were obtained on the upper and lower layers in a Beckman Model DU spectrophotometer with test tube attachment. Typical results are presented in Fig. 1. From the appearance of the curves, the separation of interfering chromogens appears to be nearly complete.

STABILITY OF COLOR

The methylene chloride-propanol extract, when not exposed to the atmosphere, shows reasonable stability for at least 30 minutes. Loss of color approximates 3% every 10 minutes with urine extracts and with the standards.

ACCURACY AND PRECISION OF TECHNIC

Twenty-nine urine specimens were split into 2 replicates which were run through the 17-KGS procedure. Before applying the Zimmermann reaction, each replicate was divided into 2 parts, one being completed by Method A and the other by Method D. The duplicates of Method A and of Method D were averaged. The results, in which the methodology was identical except for the terminal step, were

Fig. 1. Absorption curves of Zimmerman chromogens obtained in a Beckman DU spectrophotometer with test tube attachment. Chromogens were partitioned into upper and lower phases by the Demetriou extraction procedure. Solid lines, lower phase containing Zimmermann color; broken lines, upper phase containing interfering chromogens; H, urine with high 17-KS; L, urine with low 17-KS; S, dehydroepiandrosterone standard, 50 μg.
compared with respect to (a) the amount of 17-KGS found and (b) the precision as indicated by agreement between replicates.

In Fig. 2 is shown the milligrams of 17-KGS found by each of the methods in the 29 urines. The log-log relationship has been used since it was observed that the distribution of 17-KGS and of error in determination tended to be more nearly normal on the exponential scale. In Fig. 2 an identity of results with the methods is indicated by the broken line. The regression of the results of Method D on those of Method A, fitted by the method of least squares, is shown as the continuous straight line. The 95% confidence limits of the regression are also indicated. The common mean is represented by a circled point and the individual tests by x marks. It is seen that the results with the methods are essentially identical over the range tested.

With respect to the relative precision of the methods, the error between replicates was somewhat smaller with Method D than with Method A, but this lacked statistical significance. However, there is 90% confidence that the precision of Method D is not less than that of Method A.²

**CONFORMANCE TO BEER'S LAW**

Selected volumes of an ethylene chloride extract of pooled urine were dried separately under nitrogen as described above. The Zim-

²Dr. Fred Moore kindly performed the statistical analyses of the data.
merman reaction was performed on each sample, separation of brown chromogens was then accomplished as described in Method D and absorbance readings were made with a green filter (No. 54) on a Klett-Summerson filter photometer. Corrections were made for the necessary blank readings. It may be seen from the data in Fig. 3 that the results conform well to Beer’s law. This has been consistently true in experiments carried out on many different urine extracts.

ZIMMERMANN EQUIVALENTS WITH DIFFERENT STEROIDS

It was of interest to study the relative chromogenicity of certain steroids using Method A and Method D. Equivalent quantities of U.S.P. reference steroids were dissolved in alcohol and dried under nitrogen. The results are presented in Table 1.

There is good agreement between the two methods with the 17-KS used which have no ketone group in the 3 position. However, with adrenosterone and with $\Delta^4$ androstene-3, 17 dione, Method D gives lower results. The Zimmerman chromogens formed from these 2 steroids are not completely extracted into the methylene chloride-propanol layer by Method D. Part of the purple color remains in the

Fig. 3. Conformance to Beer’s law of Zimmerman colors quantitated by Method D.
Table 1. Comparison of Zimmerman Colors Formed from Different Steroids Using Method D and Method A for Quantitation*

<table>
<thead>
<tr>
<th>STEROID</th>
<th>METHOD D</th>
<th>METHOD A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEHYDROEPIANDROSTERONE</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ANDROSTERONE</td>
<td>98</td>
<td>87</td>
</tr>
<tr>
<td>3α-HYDROXY-ETIOCHOLANE-11,17-DIONE</td>
<td>148</td>
<td>140</td>
</tr>
<tr>
<td>3α-11β-DIHYDROXY-ETIOCHOLANE-17-ONE</td>
<td>79</td>
<td>82</td>
</tr>
<tr>
<td>ADRENOSTERONE</td>
<td>39</td>
<td>90</td>
</tr>
<tr>
<td>Δ¹⁶-ANDROSTENE-3,17-DIONE</td>
<td>51</td>
<td>74</td>
</tr>
<tr>
<td>CORTISONE</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Numbers represent per cent of color produced by an equal quantity of dehydroepiandrosterone.

upper aqueous phase, which explains the discrepancy between the methods. These observations are in agreement with those of Kellie and Smith (9), who demonstrated marked polarity of Zimmerman chromogens of 17-KS having a ketone group at position 3.

Numerous modifications of Method D have not been successful in preventing the adrenosterone-m-dinitrobenzene complex from passing into the aqueous layer. Interestingly, this phenomenon did not occur with 17-KS unless there was a ketone group at the 3 position. The ketone group at position 11 had no effect as indicated by the good agreement between Methods A and D with the steroid 3α-hydroxyetiocholane-11, 17-dione.

DISCUSSION

The chemical extraction technic described above (Method D) for the elimination of interfering chromogens in the Zimmerman reaction appears to be satisfactory for clinical studies. However, this method as well as the application of Allen's correction (Method A) have certain limitations. The latter method may give misleading results when applied to urine extracts high in interfering chromogens (13). Method D appears to give excellent results with such urines. However, when Method D is applied to certain 17-KS having a ketone group in position 3, it gives falsely low results and Method A is to be preferred. This effect was first noticed when performing internal
standard cortisone recoveries after sodium bismuthate oxidation. By Method D, recoveries consistently came out to be about 40\% of the theoretic value, whereas by Method A, the recoveries were approximately 90\%. It was obvious on examining the tubes from Method D that part of the Zimmerman-purple complex was in the upper aqueous layer. Since adrenosterone is the bismuthate oxidation product of cortisone (14) these results can readily be explained by examination of the data in Table I. Adrenosterone yields only about 40\% of the color with Method D as with Method A.

The phenomenon described above is not observed when these tech
nics are applied to the urine of patients receiving cortisone. Here, Method A agrees well with Method D, confirming the well-known fact that the 3-ketone group on cortisone and other related steroids is rapidly reduced in vivo to a 3-hydroxy group (12). We have not yet encountered a urine in which there was an apparent loss of Zimmerman steroid-color in the upper layer.

It has been reported recently that meprobamates will interfere with the Zimmerman reaction (15) but that the interference is eliminated with the use of the Allen correction. We have applied Method D to urines collected from patients before and after the administration of large amounts of meprobamates. No interference was detected.

SUMMARY

A simple extraction technic is described for separating interfering chromogens from the 17-KS complex formed when the Zimmerman reaction is applied to urinary extracts. The procedure is performed immediately after the Zimmerman reaction is completed. It allows quantitation of the steroid present in a filter photometer at one wave length. Results compare favorably with those obtained after dilution of the Zimmerman complex with 70\% aqueous ethanol and application of Allen's correction.

REFERENCES