ELIMINATION OF ERRORS IN THE COLORIMETRIC ASSAY OF NEUTRAL URINARY 17-KETOSTEROIDS BY MEANS OF A COLOR CORRECTION EQUATION*

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The colorimetric assay of 17-ketosteroids is based upon the fact that these substances react with the m-dinitrobenzene-alcoholic KOH reagents to give pigmented solutions (1). For crystalline 17-ketosteroids in pure solution the extinction coefficient of these solutions in the green ($E_g$) accurately reflects the 17-ketosteroid concentration according to Beer’s law (2). When this colorimetric assay procedure is applied to crude urine extracts, the $E_g$ determination is not always an accurate index of the 17-ketosteroid content, because such extracts may contain other chromogens which contribute to the $E_g$ value and hence cause an error of overestimation (2). It has been shown elsewhere that this error can be reduced by separating a portion of these interfering chromogens from the urinary 17-ketosteroids with the aid of Girard’s Reagent T (2).

The possibility of using a color correction equation for eliminating these errors in assay has been suggested by Fraser et al. (3). The equation used by them was worked out by Gibson and Evelyn (4) for another problem. It is based in principle on Vierordt’s theory that the respective concentrations of two pigments in solution may be determined by measuring the extinction coefficients of such a solution at two wave-lengths, provided the extinction-wave-length curves for each pigment are known.

As applied to the present problem, the symbols in the equation

$$(K_t \times G - B)/(K_t - K_e) = \text{corrected reading (C)}$$

have the following meaning: The extinction coefficients obtained for solutions of urine extract-m-dinitrobenzene reaction products with a green ($E_g$) and a blue ($E_b$) filter are indicated by $G$ and $B$, respectively. $K_t$ represents the ratio $E_g: E_b$ for a sample of the interfering chromogens; $K_e$ represents the ratio $E_g: E_b$ for samples of crystalline 17-ketosteroids. The corrected reading, $C$, represents a calculation of that portion of the total extinction coefficient.

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in the green (G) which is due to urinary 17-ketosteroid reaction products alone.

The value for \( K_i \) is easily determined with pure solutions of crystalline 17-ketosteroids. Ideally, the value for \( K_i \) should be determined with a pure solution of a single interfering chromogen. Because the interfering "chromogen" of urine extracts is composed of a mixture of substances whose identity has not been established, it becomes necessary to ascertain the \( K_i \) value by indirect methods. Thus, the valid application of the equation to the present problem depends upon the selection of a reasonably pure sample of the interfering chromogens of urine extracts. Samples of the interfering chromogens of different urine extracts should react for practical purposes like a single known chromogen to give pigments with essentially constant \( K_i \) values. Moreover, it is necessary to show that these interfering chromogens do not influence the 17-ketosteroid color reaction or vice versa. Except for stating that \( K_i \) was determined with extracts containing "no steroid," Fraser et al. (3) did not present data which justified the application of the equation to this problem. The purpose of the present paper is to report data showing that the equation is valid for this analysis and that it essentially obviates the need for chemical purification of neutral urine extracts prior to colorimetric assay.

**Methods**

Urine extracts were prepared and assayed according to procedures described previously (2).¹

**EXPERIMENTAL**

**Selection of Samples of Interfering Chromogens**—When crude urine extracts are treated with Girard's Reagent T, a major portion of the interfering chromogens appears in the non-ketonic fraction. These interfering chromogens give rise to colored products giving an \( E_a:E_b \) value of approximately 0.5. A smaller portion of interfering chromogens is carried over into the ketonic fraction along with the 17-ketosteroids (2). In unpub-

¹ Particular care should be taken that any ethyl ether used in the procedure is free from impurities such as peroxides. These impurities give strong color reactions with the \( m \)-dinitrobenzene-alcoholic KOH reagent. A 0.2 cc. aliquot of a 2 cc. absolute alcoholic solution of the residue obtained by evaporation of 200 cc. of ethyl ether on a boiling water bath should not give more color in the \( m \)-dinitrobenzene-alcoholic KOH reaction than 0.005 mg. of pure 17-ketosteroids. The impurities encountered in the ethyl ether can usually be removed completely by any of the standard procedures for eradicating peroxides, such as washing the ether with a 10 per cent solution of ferrous sulfate in 1 N sulfuric acid, followed by water washings.
lished experiments it has been found that these residual chromogens of the ketonic fraction are also apparently non-ketonic substances that give rise to colored products with the same $E_A:E_B$ value.

The data of Fig. 1 provide evidence which suggests that most, if not all, of the total chromogens in certain urine extracts consist of these "non-

![Graph](http://www.jbc.org/)

**Fig. 1.** The mutual relationship between the total uncorrected 17-ketosteroid values determined by colorimetric assay of partially purified ketonic fractions of 400 different 24 hour urine extracts and the color characteristics of the assay solutions as represented by the value $E_A:E_B$. The white circles are females, the black circles, males.

ketonic" substances. In Fig. 1 the values for $E_A:E_B$ of 400 urine extracts from normal and abnormal individuals of all ages are plotted against the uncorrected total 17-ketosteroid assay per day determined on partially purified ketonic fractions (2). It will be noted that the values for $E_A:E_B$ range between 2.2 and 0.6. There is a definite tendency for the lowest $E_A:E_B$ ratios to be associated with the lowest 17-ketosteroid assay values.
COLORIMETRIC ASSAY OF 17-KETOSTEROIDS

The lowest $E_o:E_B$ values observed (0.6) closely approximate the value (0.5) obtained with the non-ketonic chromogens. These low values were obtained on extracts of urine from young children and from panhypopituitary dwarfs. Such individuals presumably excrete little or no 17-ketosteroid. On the other hand, the highest values for $E_o:E_B$ (2.2) correspond to the value observed for pure 17-ketosteroids. They were obtained from patients who were suffering from corticoadrenal hyperplasia or individuals who had received injections of testosterone propionate. In these latter clinical situations the 17-ketosteroid output is known to be high. This suggests that the actual 17-ketosteroid content of extracts with $E_o:E_B$ values as low as 0.6 is probably essentially zero. Consequently the $E_o:E_B$ value, 0.6, should approximately represent the color characteristics of a "pure" solution of the interfering chromogens of urine extracts.

Behavior of Interfering Chromogens in Color Reaction—In order to find out whether the interfering chromogens alter the characteristic color reaction between 17-ketosteroids and the m-dinitrobenzene-alcoholic KOH reagents and vice versa, the $G$ and $B$ values were obtained for samples of the interfering chromogens to which known amounts of crystalline dehydroiso-

### Table I

Comparison of Determined and Theoretical Values for $E_o$ and $E_B$ before and after Addition of Dehydroisoandrosterone to Constant Amount of Pooled Interfering Chromogenic Substances

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>DHA added</th>
<th>$E_o$ Determined</th>
<th>$E_o$ Theoretical</th>
<th>$E_B$ Determined</th>
<th>$E_B$ Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>None</td>
<td>0.048</td>
<td>0.109</td>
<td>0.078</td>
<td>0.106</td>
</tr>
<tr>
<td>1B</td>
<td>0.017</td>
<td>0.108</td>
<td>0.175</td>
<td>0.105</td>
<td>0.130</td>
</tr>
<tr>
<td>1C</td>
<td>0.034</td>
<td>0.276</td>
<td>0.287</td>
<td>0.149</td>
<td>0.180</td>
</tr>
<tr>
<td>1D</td>
<td>0.145</td>
<td>0.509</td>
<td>0.517</td>
<td>0.280</td>
<td>0.286</td>
</tr>
<tr>
<td>1E</td>
<td>None</td>
<td>0.128</td>
<td>0.240</td>
<td>0.194</td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>0.024</td>
<td>0.211</td>
<td>0.219</td>
<td>0.219</td>
<td>0.321</td>
</tr>
<tr>
<td>2C</td>
<td>0.048</td>
<td>0.274</td>
<td>0.236</td>
<td>0.276</td>
<td>0.264</td>
</tr>
<tr>
<td>2E</td>
<td>0.096</td>
<td>0.426</td>
<td>0.378</td>
<td>0.347</td>
<td>0.320</td>
</tr>
<tr>
<td>3A</td>
<td>None</td>
<td>0.076</td>
<td>0.675</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>0.046</td>
<td>0.233</td>
<td>0.227</td>
<td>0.191</td>
<td>0.180</td>
</tr>
<tr>
<td>3C</td>
<td>0.093</td>
<td>0.347</td>
<td>0.364</td>
<td>0.215</td>
<td>0.242</td>
</tr>
<tr>
<td>3D</td>
<td>0.139</td>
<td>0.502</td>
<td>0.514</td>
<td>0.284</td>
<td>0.300</td>
</tr>
<tr>
<td>3E</td>
<td>0.187</td>
<td>0.638</td>
<td>0.653</td>
<td>0.342</td>
<td>0.373</td>
</tr>
<tr>
<td>3F</td>
<td>0.290</td>
<td>0.886</td>
<td>0.930</td>
<td>0.438</td>
<td>0.499</td>
</tr>
</tbody>
</table>
androsterone-17 had been added. The determined values for \( G \) and \( B \) (Table I) may be compared with the theoretical values. The latter were calculated by adding to the \( G \) value and \( B \) value obtained with interfering chromogens alone the \( G \) and \( B \) values corresponding to the amount of pure dehydroisoandrosterone-17 added. The results show that the determined and theoretical figures agree reasonably well within the limits of experimental error. These experiments thus indicate that the reactions of

![Diagram](http://www.jbc.org/)

Fig. 2. The mutual relationship between the ratio for corrected to uncorrected 17-ketosteroid assay values and the value for \( E_G : E_B \). The white circles describe the curve which is calculated from the color correction equation. The crosses, black circles, and triangles represent points determined by Experiments 1, 2, and 3, respectively, of Table I.

17-kctosteroids and of the interfering chromogens with the \( m \) dinitrobenzene reagents are the same in mixtures as in separate solutions.

**Accuracy of Color Correction Equation**—With the aid of Gibson and Evelyn's equation, the value of the corrected 17-ketosteroid assay \( (C) \) has been calculated for theoretical solutions with a constant \( G \) value and \( E_G : E_B \) values ranging between 0.6 \( (K_i) \) and 2.2 \( (K_s) \). The ratio of the corrected \( (C) \) over the uncorrected \( (G) \) values plotted against the respective \( E_G : E_B \) ratio of each theoretical solution gives the curve described by the white circles in Fig. 2. The other symbols in the figure represent similar values determined experimentally (Table I). It was assumed that
the pooled samples of interfering chromogenic substances contained no 17-ketosteroid. Thus the ratios for corrected to uncorrected 17-ketosteroid values in experiments designated A is zero. In the experiments designated B to F, the ratios for corrected to uncorrected 17-ketosteroid contents were determined by dividing the value corresponding to the known amount of pure 17-ketosteroid added by the value determined by the uncorrected reading \( G \). The \( E_A \) and \( E_B \) values for each experiment were used to determine the \( E_A : E_B \) ratio. The closeness with which the theoretical

![Graph](http://www.jbc.org/)

Fig. 3. Comparison of results obtained when the color correction equation is applied to assays made on crude and on partially purified extracts obtained from the crude extract, respectively. The common denominator is the uncorrected value for the crude extract. The interrupted curve represents the points where all values would fall if the two corrected values were identical.

and experimental values approach the same curve substantiates the validity of the equation and the theoretical considerations upon which it is based.

Substitution of Color Correction Equation for Partial Purification Procedure—The ratio of corrected to uncorrected 17-ketosteroid assay values has been determined as outlined above on twenty-four separate crude neutral extracts of urine. Subsequently, the crude extracts were treated with Girard's Reagent T (2) and the corrected 17-ketosteroid assay value of the partially purified ketonic fraction was obtained. The abscissa of Fig. 3
gives the ratio of corrected to uncorrected 17-ketosteroid values determined for the crude extract. The ordinate represents the ratio of the corrected ketonic value to the uncorrected crude value. It will be seen that with one exception the two ratios locate points which fall reasonably close to the theoretical dotted line describing the ideal situation in which both ratios are exactly the same. In the exceptional case, marked by a circle, the correction on the crude value was insufficient. It was observed that this particular crude extract was unusually pigmented. These pigments, which are also known to cause an overestimation in the assay (5), were largely eliminated when the crude extract was purified with Girard's reagent.

Comments

It is clear from the foregoing experimental observations that the non-ketonic chromogens must be taken into account in the colorimetric assay of urinary 17-ketosteroids. Relatively speaking, they may be responsible for much greater errors in the quantitative assay of urinary 17-ketosteroids than any of the other factors which have been studied in this (2, 5) or other laboratories (6–9). The data presented here show that the error introduced by the presence of these substances can, with rare exceptions, be eradicated by means of the color equation of Gibson and Evelyn as suggested by Fraser et al. (3).

Application of the equation is easy once the values for \( K_i \) and \( K_s \) have been determined. If reagents and colorimeter light filters similar to those used here are employed, it should be possible to utilize the values for \( K \) described herein. It is more advisable, however, for each laboratory to determine its own \( K \) values. For this purpose, a crystalline 17-ketosteroid and a representative pooled sample of urine extract containing no 17-ketosteroid (such as a non-ketonic fraction from a crude urine extract) are all that is required. Once these are established, a convenient table listing the ratios of corrected to uncorrected 17-ketosteroid values (see Fig. 1) for the entire range of \( E_o : E_B \) values may be drawn up for routine use. This table serves as a list of correction factors corresponding to each \( E_o : E_B \) value.

SUMMARY

Evidence is presented which shows that interfering chromogenic substances may cause significant and variable errors of overestimation in the colorimetric assay of neutral urinary 17-ketosteroids. Except in unusual instances, these errors may be largely eliminated by means of a simple color correction equation without preliminary chemical purification of the crude neutral extract.
We are indebted to Dr. A. M. Butler for suggestions in the preparation of this paper.

BIBLIOGRAPHY

CORRECTION
On p. 211, Vol. 143, No. 1, March, 1942, lines 2 and 3 from the bottom, read $\eta : E_0$ for $E_0 : E_B$. 
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