Characteristics of a 17-Ketosteroid Reaction

L. A. Kraushaar, E. Epstein, and B. Zak

An improved method for the determination of 17-ketosteroids is described, which involves an increase in sensitivity by the simple expedient of upsetting the equilibrium of the reaction in removing the products of reaction by precipitating the chromophore formed. This precipitation system appears to achieve about 1.6 times the color sensitivity for the same reaction carried out as a solution process. An alternative extraction step for color purification is described. Spectrums, variabilities in reagent parameters, and statistical evaluations are discussed.

An improved version of the Zimmermann modification (1) of a methylene ketone reaction with m-dinitrobenzene has been described for the determination of 17-ketosteroids (17-KS) (2). The application to automation (3) and chromatography (4, 5) were subsequent developments of this technic. A study was carried out on the parameters of the reaction, and it will be shown that further improvements in its characteristics are possible because it appears that the reaction conditions, though useful as described (2, 3), were still not optimal.

The results obtained in this study are in keeping with the stated purposes which are as follows:

1. To overcome what appears to be the unfavorable equilibrium characteristics of the color reaction
2. To reduce the variability in the day-to-day standardization by increased color stability
3. To improve considerably the sensitivity of the color reaction to a maximum
4. To offer the alternative of purification of color by a simple extraction process
5. To speed up the color reaction and decrease its light sensitivity
6. To reduce the use of organic solvents in reagents and color reaction to a minimum.

From the Department of Pathology, Beaumont Hospital, Royal Oak, Mich., St. Joseph Mercy Hospital, Pontiac, Mich., and Wayne State University School of Medicine, Detroit, Mich. 48207.

Supported in part by the Detroit General Hospital Research Corporation.

Received for publication Sept. 20, 1965; accepted for publication Jan. 25, 1966.
Material and Methods

Reagents

**Hyamine 1622 (2.5%)**  Dissolve 5.0 gm. of H-1622 (Rohm & Haas Company) in 200 ml. of distilled water.

**m-Dinitrobenzene (MDB) (0.18%)**  Dissolve 0.36 gm. of MDB in 200 ml. of the 2.5% H-1622 solution.

**Potassium hydroxide (10N)**  Dissolve 165 gm. of KOH in distilled H2O and dilute to 250 ml.

**Stock dehydroepiandrosterone standard (1.0 mg./ml. of methanol)**

**Working dehydroepiandrosterone standard (50 μg./ml. of methanol)**

**Sodium hydroxide (50% w/w)**

**Methanol (spectral grade)**

**1,1,1-Trichloroethane or ether**

**Xylene**

Procedure

**Hydrolysis**

Pipet 5.0 ml. of filtered urine into a 25- × 150-mm. test tube, add 0.015 ml. of formalin and 0.5 ml. of concentrated HCl. Cover the tubes with a perforated snap cap, and place the tube in a boiling water bath for 15 min. (6). Cool the tube in an ice bath for 3 min.

**Extraction and Evaporation**

Add 25 ml. of 1,1,1-trichloroethane to each tube, cap, and mix them on a Vortex mixer for 1 min. Centrifuge at 2500 r.p.m. to clarify the 2 phases. Aspirate off the aqueous phases, add to the remaining organic phases 5 ml. of 50% NaOH, cap, mix, and centrifuge again. Remove the aqueous phases and filter the organic phases. Evaporate two 10-ml. aliquots of each extract to dryness.

**Color Development**

Add 0.5 ml. of methanol to each tube, followed by 1.0 ml. of 10N KOH and 0.2 ml. of MDB reagent. A precipitate forms. After 15 min., add 2.0 ml. of 2.5% H-1622 to dissolve the precipitate. Read the colored solution in the spectrophotometer at 525 mμ.

**Color Extraction**

Add 3 ml. of xylene and extract the phase containing the precipitate as noted above. Centrifuge the liquids to clear the xylene phase. Transfer this layer to a cuvet, and read at 525 mμ against a reagent blank similarly prepared.
Discussion and Results

When a substance with low solubility is formed by a chemical reaction, the greater part of the substance will leave the solution, and the reaction will go to completion. The present work is an excellent example of the use of a reaction of this type, which appears to be at approximately two-thirds equilibrium when it is normally carried out as a solution process (2). If the product of the methylene ketone group reaction with MDB could be forced out of solution as a precipitate or an oil, as it is in this case, then the equilibrium would be upset and the reaction continued until all the 17-ketosteroid was consumed. Simple solution of the precipitated material by the further addition of solubilizing reagent, H-1622, would be all that would be necessary to achieve measurement of a uniform chromophoric system. Such a process would obviate changes in amount of color due to changes in the alkalinity of the reagent with time or method of preparation. As long as the potassium hydroxide was in a plateau region for precipitation of the colored complex, the amount of color yield should not vary. The absorptivity at 525 m\(\mu\) calculated for a DB spectrophotometer\(^*\) was 64.6 L./gm. cm., and this can be reproduced repeatedly. There was no day-to-day variability in the amount of absorbance obtained by the described procedure for a given concentration of ketosteroid. Since the solution technic (2) has an absorptivity of 39.7 L./gm. cm. at the 525 m\(\mu\) peak, it can be seen that the color yield has been increased by 60% of the original, and this represented a substantial change in sensitivity.

Another advantage was the decrease in time required for full color formation. There was no difference in absorbance per unit concentration of 17-ketosteroid for color development in a period of 5 min. to well over an hour. In addition, the color can be developed under regular lighting conditions, an indication that there are no undesirable photosensitive properties.

The use of H-1622 as solubilizer for an adequate amount of MDB in aqueous solution eliminated the need to use ethanol or methanol in all phases except the solubilization of the urine residue. This obviated purification of ethanol, which is commonly used, while methanol can always be obtained in very adequate spectral grade.

Figures 1 and 2 show the time factor involved in the color reaction. When the progression of the stated reaction was followed, plateau color formation was achieved in 5 min. This was in contradistinction to the solution technic previously described (2) where the equilibrium

\(^*\)Beckman Instruments, Inc., Fullerton, Calif.
was approached in 15 min., but a slow increase in absorbance continued over the next 2 hr. Figure 1B shows the effect of varying the present H-1622 used in the reaction. A plateau was reached at a very low concentration, and any concentration between 0.5 and 5.0% functioned adequately. A third variable is shown in Fig. 1A where two facts are obvious. The first is related to the linearity of reaction, and the second, to the plateau for MDB for 60 and 100 μg/ml. of dehydroepiandrosterone standard. The reaction was linear, obeying the Beer-Lambert Law when the plateau was reached. The concentration of MDB required

---

**Fig. 1.** MDB and H-1622 concentrations. Molar combined ratio, MDB:17-KS = 1:1; H-1622:17-KS = 1:1. A. Plateau for MDB concentration. B. Plateau for H-1622 concentration. Fig. 2. Plateau for time of color formation.
was in a safe range when it was kept at or near the saturation point of 0.2%. The calculations found on continuous variation of each variable while the other variables were kept constant indicated that the ratio of MDB to H-1622 to ketosteroid was 1:1.

Figures 3–6 show the spectral characteristics of the described reaction for standards, urine residues, and appropriate blanks. In addition, a comparison was made of the spectrums for the present system, which shall be referred to as the precipitation technic, to the spectrums

![Diagram of spectral characteristics](image)

**Fig. 3.** Standards spectrums for solution and precipitation technics. *A* indicates curve for standard versus reagent blank of new method; *B*, curve for standard versus reagent blank of older method (*S*); *C*, curve for reagent blank versus water, precipitation technic. **Fig. 4.** Urine spectrums for precipitation and solution technics. *A* indicates curve for urine extract versus reagent blank, precipitation technic; *B*, solution technic; *C*, reagent blank versus water; *D*, urine blank versus water. **Fig. 5.** Absorbance of xylene-extracted standard. *A* indicates spectrum prepared with xylene extract of standard color; *B*, xylene-extracted aqueous phase of standard versus water; *C*, extracted reagent blank versus xylene. **Fig. 6.** Absorbance of xylene-extracted urine. *A* indicates spectrum obtained with xylene-extracted urine; *B*, aqueous phase, urine test after xylene extraction; *C*, reagent test blank; *D*, urine test blank versus xylene.
of the solution system previously described, (2) which shall be referred to as the solution technic.

In Fig. 3, the contrast between the single-peaked bell-shaped curve for the standard in the precipitation technic (Curve A) and the two-peaked Curve B for the solution technic demonstrates that the difference in sensitivity of the two methods is striking and obvious.

With urine extract (Fig. 4), there is again a marked difference between Curves A and B. There has been a shift in the peak wave length for the solution technic, standard as opposed to urine extract, whereas the precipitation technic shows the same peak wave length for both mediums. It has previously been pointed out (7) that very few urinary extracts show the same spectrums for both urinary extracts and standards, and as a result, mathematical corrections for the concomitant brown color have been formulated to overcome this.

The spectrums shown in Fig. 5, xylene extract of standard color, and Fig. 6, xylene-extracted urine, clearly indicate that the extraction technic eliminates the yellow background arising in both samples and standards.

![Fig. 7. Regression equation for known recoveries with plotted present values versus found values. Range covered, 2.5-30.0 mg./L.; range found, 2.5-30.5 mg./L.; standard deviation, ± 0.26 mg./L.]

The similarity of the spectrums in the precipitation technic for standard and sample is in contradistinction to the small but significant spectral shift which takes place in the solution technic. In addition, because of the presence of the shoulder in the solution technic at 420 mμ,
which is absent from the spectrums of the procedure described in this report, one might infer that the shoulder is present because of the equilibrium reaction.

Figure 7 shows the linear correlation of present and found values for absolute concentrations of dehydroepiandrosterone as determined by the following equation.

\[ Y = a + bX \]
\[ Y = 0.01 + 1.01X \]

The estimating equation shows a slope closely approximating 1, with a standard deviation of ±0.26 mg./L. The excellent data indicated that the analytical measurement device was accurate for this type of controlled experimentation. The plotted points scattered along the line of the estimating equation are a representative group of analytical findings when the described precipitation technic was applied to known quantities of 17-KS as well as several xylene-extraction recoveries.

References