Simplified 2,6-Di-*tert*-butyl-*p*-cresol Colorimetric Method for Unsaturated-3-keto Steroids

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An improved modification of the original 2,6-DTBPC method for the determination of unsaturated 3-keto steroids has been developed. The presented technique features a simpler mode of operation, improved sensitivity, equivalent reproducibility, and extension of the original method to the analyses of 6-fluoro 3-keto steroids unsaturated at both the 1 and 4 positions. Limited application to pharmaceutical dosage forms is also discussed.

SINCE THE APPEARANCE of the original procedure (1) describing the use of 2,6-di-tertiary-butyl-*p*-cresol (DTBPC) as a reagent for the assay of unsaturated 3-keto steroids, several publications have been presented as proposed improvements of the original technique. Ansari's modification (2)—the use of freshly prepared and standardized 1.0 *N* sodium hydroxide in place of nonstandardized 5% alkali and 0.5% DTBPC in 90% ethanol rather than 0.6% in 95% ethanol—retained the desired specificity and eliminated the cumbersome heating and stirring apparatus initially described.

Bianchi's technique (3), though eliminating the use of elevated temperature for color development, resulted in a loss of specificity. Steroids containing a 4-unsaturated-3-keto group showed maxima between 380 and 385 mμ, irrespective of the substituent at the 11 position, while the original method presented distinct maxima at 471 and 625 mμ. Cross (4) also reported maxima between 373 and 375 mμ for the above type steroids by merely heating the steroid with alkali and without the incorporation of DTBPC reagent.

Bartos (5), reasoning that the agitation of reactants merely served to oxidize the DTBPC and that an oxidized form of DTBPC was the actual reagent, sought simply to include 0.03% hydrogen peroxide and successfully eliminated the necessity of agitating the reactants during color development. Maxima were reported at 460 mμ (4-unsaturated-3-keto steroids with an 11-keto group), at 500 mμ for saturated-3-keto steroids, and at 610 mμ for 4-unsaturated-3-keto steroids with a hydroxylated or unsubstituted 11 position. Bartos' modification was the first to reveal that 1,4-unsaturated-3-keto steroids would react with DTBPC, though at reduced sensitivity.

The present method permits color development within 14 to 21 minutes at 90 to 91°, avoids the necessity of having to stir the reactants during color development, does not require the use of hydrogen peroxide, results in greater sensitivity (40 to 80 mcg. of steroid) than the original method, retains the original specificity, and does not require the use of standardized alkali. The method has, however, limited applicability for the analyses of formulated steroids.

EXPERIMENTAL

Apparatus.—An improvised water bath with all component parts is described in Fig. 1. Test tubes, culture type with screw caps, 20 × 125 mm. and a microliter syringe with 50 μl. capacity were also used.

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Reagents.—The following reagents were used: *N*-butyl alcohol (Baker's analyzed reagent grade); DTBPC reagent, 2,6-di-tertiary-butyl-*p*-cresol (Eastman practical grade or Koppers' technical or food grades) 0.80% in *n*-butyl alcohol; TMAH reagent, 10% aqueous tetramethyl ammonium hydroxide (Eastman); and steroid standards, 40 mcg. of reference standard material per 1.0 ml. of *n*-butyl alcohol.

Unformulated Steroid Assay Procedure.—Using a semimicro balance, weigh to the nearest 0.02 mg. about 10 mg. of assay sample into a 50-ml. volumetric flask. Add about 30 ml. of *n*-butyl alcohol. Stopper and shake to dissolve the steroid. Dilute to the mark with solvent. Pipet 5.0 ml. of the above solution into a 25-ml. volumetric flask and dilute to the mark with *n*-butyl alcohol. Pipet 2.0 ml. of the final dilution (*ca.* 80 mcg. of steroid) into a 20 × 125-mm. culture tube.

Prepare (as above) a reference standard solution of the same steroid equivalent to about 80 mcg. of steroid per 2.0 ml. of *n*-butyl alcohol. Pipet 2.0 ml. of this solution into a second culture tube of the same dimensions as above. Add 2.0 ml. of *n*-butyl alcohol to a third culture tube which is to serve as a reagent blank.

To each of the three culture tubes add 3.0 ml. of 0.80% DTBPC in *n*-butyl alcohol, followed by the addition of 50 μl. of 10% aqueous TMAH with a 50-μl. syringe. Replace the screw caps on each of the three tubes.

Immediately insert each tube into the 20-mm. apertures in the aluminum support plate, resting on the 400-ml. beaker containing 200 ml. of magnetically stirred 90 to 91° water (Fig. 1). (The fourth opening in the plate is used to accommodate a thermometer inserted in a No. 3 rubber stopper.) The position of each Pb tube-guide should be preset (prior to the addition of reactants to the culture tubes) to allow just the bottom edges of the tubes to clear the rotating magnetic bar. The Pb strips also serve to offset the buoyant effects of the water bath on the inserted tubes.

Heat for 14 to 21 minutes at 90 to 91°, depending

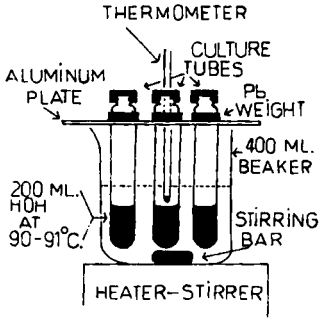

Fig. 1. — Diagram of the improvised water bath used to develop the steroid–DTBPC colors.

TABLE I.—DATA AND CONDITIONS FOR OPTIMUM DTBPC COLOR REACTION ACCORDING TO TYPE OF STEROID

Steroid	Type[a]	Max. mμ	Absorbance, 1-cm. path, *vs.* Reagent Blank	Mcg.	Heating Time, Min.
Fluocinolone acetonide	A	620	0.507, 0.503	80	21
			0.508, 0.513		
			0.258, 0.257	40	21
21-Dihydrogen phosphate monosodium salt of above compound	A	620	0.363, 0.363	80	21
Paramethasone acetate	A	620	0.292, 0.292	80	21
			0.292, 0.299		
			0.296		
Progesterone	B	620	0.474, 0.474	80	21
			0.474		
Hydrocortisone acetate	B	630	0.697, 0.736	80	21
			0.716		
Cortisone acetate	C	485	0.322, 0.322	80	14
			0.322		

[a] Type A steroids, those which feature a 6α-fluoro-11β-hydroxy-3-keto (1,4-unsaturated) structure; Type B steroids, those which show a 11-unsubstituted or 11-hydroxylated-3-keto (4-unsaturated) structure; Type C steroids, those having a 3,11-diketo (4-unsaturated) structure.

on the type steroid (Table I) being analyzed. Immediately immerse all three tubes in an ice bath at 0° for 3 minutes. Determine the 1-cm. absorbance at the particular analytical wavelength (Table I) of the steroid during the subsequent 15-minute interval, using as a reference the simultaneously prepared reagent blank. For steroids, of other types than for which data are presented, the analytical wavelengths and optimum heating times should be determined by the analyst.

Per cent purity = $A_p/A_s \times S/W \times 100$, where A_p and A_s are the respective 1-cm. absorbance values of the analytical sample and the steroid reference standard and where the respective microgram weights of the analytical sample and steroid standard in the colored solutions are W and S.

Formulated Steroid Assay Procedure.—A portion of a filtered chloroform extract of the steroid equivalent to 200 mcg. of steroid is evaporated to dryness in a 50-ml. glass-stoppered flask. The residue is stirred for 5 minutes with 5.0 ml. of *n*-butyl alcohol. Exactly 2.0 ml. of this solution, containing about 80 mcg. of steroid, is transferred to a culture tube and the color developed as above along with 80 mcg. of corresponding reference standard steroid and the reagent blank. The optimum heating time and analytical wavelength, being dependent on the type steroid, should either be predetermined or based on the data provided in Table I.

Per cent steroid = $A_p/A_s \times B/D \times C/E \times 0.00025$, where A_p and A_s remain as defined above, B is the mcg. weight of steroid standard per 2.0 ml. of *n*-butyl alcohol, C is the volume (ml.) of chloroform used to extract E ml. or E Gm. of formulation, and D is the volume (ml.) of chloroform extract evaporated to dryness and equivalent to 200 mcg. of steroid.

RESULTS AND DISCUSSION

Analyses of a tablet formulation containing 0.666% 6α - fluoro - 11β,17α,21 - trihydroxy - 16α - methylpregna - 1,4 - diene - 3,20 - dione - 21 - acetate (paramethasone acetate), led to assay values of 0.660, 0.650, and 0.644% steroid. These results correspond to an average value of 0.651% paramethasone acetate, with a mean average deviation of ±0.0054% steroid. A placebo (cornstarch, lactose, magnesium stearate, polyvinylpyrrolidone, and FD & C No. 4 and 5 dyes) gave no color at 625 mμ. Direct ultraviolet analyses (methanol at 240 mμ) of residues from chloroform extracts of these tablets led to a mean assay value of 0.669% steroid, using as a reference a correspondingly treated placebo tablet.

Scope of Reaction.—1-unsaturated-3-keto steroids (480 to 485 mμ), prednisolone (590 mμ), and prednisone (575 mμ) produced maxima of weak and irreproducible intensities. The introduction of 6α-chlorine to the prednisone molecule failed to enhance its reactivity toward the DTBPC reagent. The types of 3-keto steroid which gave the most sensitive and reproducible responses are presented in Table I.

Temperature Conditions.—No other temperatures were investigated beyond the 90–91° range used in the development of this method.

Heating Time.—The optimum reaction time at 90–91° for 3,11-diketo steroids (unsaturated at the 4 position) was established to be between 13 and 14 minutes; while 20 to 21 minutes were required for 6α-fluoro-11-hydroxy-3-keto steroids (unsaturated at both the 1 and 4 positions), for 11-hydroxy-3-keto steroids (4-unsaturated), and for 11-unsubstituted 3-keto steroids unsaturated at the 4 position.

DTBPC Concentration.—The concentration of DTBPC was varied from 1.0 to 0.60%, and colors were developed keeping the other variables constant. Increasing the concentration of reagent above 0.8% tended to increase absorbance values for the steroids examined, but led to less reproducibility of color intensity. Concentrations less than 0.8% DTBPC resulted in decreased color intensity and decreased reproducibility.

TMAH Concentration.—Increasing the volume of 10% TMAH beyond the recommended 50-μl. level led to lack of reproducibility. Levels of 100, 150, and 200 μl. of 10% TMAH were tested under the reaction conditions.

Color Linearity.—The chromogen for fluocinolone acetonide (620 mμ) was linear within the 40–80-mcg. range. Linearity of color for the other steroids was not evaluated over different concentration ranges.

Reproducibility.—The data presented in Table I indicated that the technique is suitable if a control reference standard is analyzed along with the analytical sample. The paired absorbance values

in this table represent simultaneous but not independent duplicate determinations. Each distinct paired group (or single value) represents a determination independent of the other group. Intensities of the developed chromophores in each instance exhibited a mean average deviation of ±2% or less.

Color Stability.—In all instances the color of the steroid chromogen was stable during the 15-minute interval after the cooling step. Longer time intervals were not investigated.

Interferences.—Chloroform soluble carboxylic acids (acetylsalicylic acid and stearic acid, etc.) and esters (sorbitan monostearates, methyl, and propyl parabens, etc.) tended to inhibit color formation. Consequently, cream formulations were not capable of being assayed using the described reagent.

Reagent Blank.—The 1-cm. absorbance values at 485 and 620 mμ for the reagent blank were, respectively, 0.116 and 0.008.

General Aspects.—DTBPC reagent, oxidized by refluxing and aerating for 1 hour, gave identical color intensity for fluocinolone acetonide (6α,9α-difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide) as did untreated reagent, indicating that an oxidized form of DTBPC is not necessarily the reactant.

Paper chromatograms of fluocinolone acetonide, impregnated and heated with the combined TMAH–DTBPC reagent, indicated that this reagent is not suitable for the detection of steroid spots.

Fluocinolone acetonide color obtained by the original method (1) was lower by a factor of 7.5 relative to this method. The former method was, however, 1.6 times more sensitive for 3, 11-diketo (4-unsaturated) steroids than the present method.

From the data presented the following conclusions may be made: (*a*) that type A steroids (Table I) containing a 6α, 9α-difluoro moiety (fluocinolone acetonide) produced more color than their 6α-fluoro counterparts (paramethasone acetate), and (*b*) that the molar response of 11-unsubstituted-3-keto (4-unsaturated) steroids toward the DTBPC reagent was decreased by substitution of an 11-keto function and increased by the introduction of a 11-hydroxyl group.

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Assay for Methapyrilene in Complex Mixtures

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A method is presented for the estimation of methapyrilene in complex mixtures which may include other antihistamines. Briefly, it consists of treating ninhydrin with methapyrilene which has been extracted from an alkaline solution with petroleum ether.

ANTIHISTAMINES have proved their therapeutic value over the years. Methapyrilene is one of the established antihistamines which has found increased usage by itself and in combination with other ingredients.

Many methods exist for the estimation of methapyrilene. The Association of Official Agricultural Chemists (1) and "The National Formulary" (2) describe methods employing an ultraviolet absorption. Clair and Chatten (3) describe a nonaqueous method and Cox *et al.* (4) employ chromatography and electrophoresis. Another method described by Celeste and Turczan (5) uses gas chromatography after a preliminary extraction. This article is concerned with a spectrophotometric method for its determination, particularly when it is present in pharmaceutical dosage forms with other materials.

EXPERIMENTAL

Materials.—Reagents used in the experiments were of analytical grade where available and used without additional purification. The following materials were used: ninhydrin (1,2,3 triketohydrindene) (Eastman Kodak); petroleum ether; sulfuric acid; polysorbate 80 U.S.P.; methapyrilene hydrochloride N.F.; sodium hydroxide; phenylephrine hydrochloride U.S.P.; dextromethorphan hydrobromide; chlorpheniramine maleate U.S.P.; and tripelennamine hydrochloride U.S.P.

Apparatus.—Beckman model DU spectrophotometer was used.

Procedure.—The method is applicable to liquid or solid dosage forms. A portion of liquid containing about 5 mg. of methapyrilene hydrochloride is transferred to a separator. The solution is rendered alkaline with either 1 *N* sodium hydroxide solution or sufficient sodium bicarbonate. The alkaline solution is then extracted successively with 15 ml. and 3 × 10 ml. of petroleum ether. The petroleum ether extracts are passed through a cotton plug moistened with petroleum ether into a 50-ml. volumetric flask. After rinsing the separator with about 3 ml. of solvent and filtering into the flask, the liquid is brought to the mark and mixed. One milliliter of the solution, equivalent to about 100 mcg. of antihistamine, is carefully transferred to a 10-ml. flask and the petroleum ether removed with a gentle stream of nitrogen for about 5 minutes. To the residue is then added 0.25 ml. of a 1% solution of 1,2,3 triketohydrindene (ninhydrin) in concentrated sulfuric acid. This solution should be freshly prepared, but is usable for several days. The flask is rotated so that the liquid covers its surface to insure complete contact with the residue. After about 5 minutes of intermittent rotation, 5 ml. of a 5% solution of poly-

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