Triphenyltetrazolium Determination of 17,21-Dihydroxy-20-ketosteroids in Tablets

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The recommended color development conditions of Johnson, King, and Vickers (1) for the triphenyltetrazolium determination of bulk hydrocortisone have been applied to the tablet dosage forms of prednisone and prednisolone.

THE U.S.P. XVI method (2) for the assay of the 17,21-dihydroxy-20-ketone side chain of steroids is the Mader-Buck (3) procedure. During the application of this method to tablets, it was noted that the variations obtained were greater than might have been anticipated. The recent publication of Johnson, King, and Vickers (1) using hydrocortisone bulk powder had conclusively demonstrated some of the sources of variance and had outlined a procedure to overcome them. The utilization of the Johnson, King, and Vickers color development conditions to the tablet dosage form of 17,21-dihydroxy-20-ketosteroids therefore seemed desirable.

EXPERIMENTAL

Reagents.—U.S.P. 95% ethanol. Triphenyltetrazolium chloride reagent solution; dissolve 50 mg. of 2,3,5-triphenyltetrazolium chloride in 10 ml. of U.S.P. ethanol, this solution should be freshly prepared and kept protected from light. Tetramethylammonium hydroxide reagent solution; dilute 5 ml. of 10% aqueous solution to 100 ml. with U.S.P. ethanol. Chloroform. Dry nitrogen.

Tablet Extraction.—The steroid is extracted from the tablets by suspending a crushed aliquot of the tablets in water and shaking with multiple portions of chloroform as directed in U.S.P. XVI. A 10-ml. aliquot of the chloroform extract is evaporated in a 50-ml. nitrogen-flushed low-actinic glass volumetric flask (or a glass-stoppered Erlenmeyer flask) under a stream of nitrogen, on a steam bath. Heating with nitrogen is continued for 3 minutes after dryness is observed.

Color Development.—After the addition of 20 ml. of U.S.P. alcohol, the stoppered flask is mechanically shaken for 15 minutes, 2 ml. of triphenyltetrazolium chloride reagent solution is added, followed by 2 ml. of tetramethylammonium hydroxide reagent solution. The surface of the solution is swept with nitrogen for 15 seconds, the flask immediately stoppered, and placed in a 30° water bath. The reaction is allowed to proceed for 45 minutes for prednisone (Meticorten) tablets and 60 minutes for prednisolone (Meticortelone) tablets. The absorbance at 485 mμ is measured against a reagent blank.

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RESULTS AND DISCUSSION

The application of the U.S.P. XVI procedure for the assay of 17,21-dihydroxy-20-ketosteroids has resulted in a large variation both in the results obtained in any given day as well as those obtained from day to day. Very poor reproducibility is often obtained in duplicate determinations of the standards themselves. Table I shows the results obtained by two analysts on five batches of tablets assayed on five consecutive days.

The major differences between U.S.P. XVI and the Johnson, King, and Vickers procedure are the use by the latter of low actinic glassware, removal of air in the reaction flasks by nitrogen, use of a 30° bath for the color development, and the reversal of the order of addition of the two reagents, tetramethylammonium hydroxide and triphenyltetrazolium chloride.

Before proceeding with the tablet assays, the effect of the variables studied by Johnson, *et al.*, were re-examined using prednisone and prednisolone standards.

Effect of Alcohol.—Anhydrous ethanol and 95% ethanol are the solvents used in most published procedures (1–5). The effects of different alcohols both on the steroid and on the reagent blanks are shown in Table II. It is not known whether the exceedingly high values of the blank readings obtained with isopropanol and *t*-butanol are due to the alcohols themselves or to some possible contaminant in them. Ethanol is the best alcohol to use in this procedure.

Effect of Water.—Increasing the water content in ethanol decreased the rate of color development. However, when longer development times were used, the absorbance readings were comparable to those obtained with the anhydrous ethanol. Consequently, the use of U.S.P. 95% ethanol was found to be very satisfactory.

Effect of Contaminants.—Low absorbance values were obtained in the presence of air, oxygen, and acetic acid. If these were present in relatively large amounts, the color development was inhibited. The presence of small amounts of chloroform and benzene did not interfere with the reaction Small amounts of acetaldehyde increased the absorbance readings.

Effect of Tetramethylammonium Hydroxide Concentration.—Both the U.S P. XVI and the Johnson, King, and Vickers procedures specify the same concentration of tetramethylammonium hydroxide. When this concentration was halved, the rate of color formation was identical but higher absorbance

TABLE I.—ANALYSIS OF PREDNISOLONE TABLETS BY THE U.S.P. XVI PROCEDURE[a]

Sample	Absorbance											
	Analyst A						Analyst B					
	Std. 1	Std. 2	Tab. 1		Tab. 2		Std. 1	Std. 2	Tab. 1		Tab. 2	
A	0.403	0.381	0.377	0.350	0.363	0.368	0.394	0.392	0.382	0.403	0.388	0.400
B	0.383	0.388	0.348	0.345	0.357	0.355	0.400	0.405	0.348	0.357	0.367	0.370
C	0.390	0.373	0.425	0.422	0.378	0.374	0.395	0.383	0.382	0.386	0.386	0.387
D	0.403	0.380	0.387	0.383	0.390	0.387	0.410	0.409	0.399	0.408	0.411	0.408
E	0.381	0.390	0.393	0.395	0.395	0.394	0.403	0.404	0.411	0.415	0.402	0.406

[a] Steroid concentration = 5 mg. per tablet.

TABLE II.—EFFECT OF VARIOUS ALCOHOLS ON ABSORBANCE VALUES[a]

Alcohol	Absorbance	
	Blank	Steroid
Anhydrous ethanol	0.050	0.470
S. D. 3A, 95% ethanol	0.045	0.430
U.S.P. 95% ethanol	0.040	0.407
Methanol	0.010	0.090
Isopropanol	0.590	0.700
t-Butanol	0.820	1.510

[a] Prednisone concentration = 0.25 mg. per 24 ml.

TABLE III.—EFFECT OF BASE STRENGTH ON THE RATE OF COLOR DEVELOPMENT[a,b]

Development, min.	Absorbance	
	Base 1:10 Dilution	Base 1:20 Dilution
15	0.411	0.410
30	0.408	0.420
45	0.410	0.425
60	0.407	0.420
75	0.402	0.420

[a] Prednisone concentration = 0.25 mg. per 24 ml. [b] Temperature for color development was 25°.

TABLE IV.—EFFECT OF TIME BETWEEN REAGENT ADDITIONS[a]

Time Between Additions, min.	Absorbance	
	Base[b] Added First	Stain[c] Added First
0.5	0.400	0.340
5	0.310	0.380
10	0.272	0.370
15	0.214	0.365
20	0.180	0.380

[a] Prednisone concentration = 0.25 mg. per 24 ml. [b] Tetramethylammonium hydroxide. [c] Triphenyltetrazolium chloride.

values were obtained as well as slightly improved color stability as shown in Table III.

Order of Reagent Addition.—The order of addition of tetramethylammonium hydroxide reagent solution and triphenyltetrazolium chloride reagent solution was relatively unimportant when only a few samples were run. However, when multiple samples were run, such as is usual in a quality control laboratory, the addition of one reagent to all the reaction flasks before the next reagent addition may introduce a 5 to 10-minute interval between the addition of the tetramethylammonium hydroxide and the triphenyltetrazolium chloride. In this case, the order of reagent addition becomes very important. If triphenyltetrazolium chloride was added first to each reaction flask, no difficulty was encountered. However, if the tetramethylammonium hydroxide was added first, drastic reductions in absorbance values were encountered as the time interval between reagent additions was increased (Table IV), when using identical color development times measured from the time of addition of the last reagent.

TABLE V.—EFFECT OF TIME ON COLOR STABILITY[a,b]

Time, min.	Absorbance	
	Prednisone	Prednisolone
15	0.415	0.310
30	0.430	0.430
60	0.423	0.455
90	0.425	0.455
120	0.422	0.455

[a] Steroid concentration = 0.25 mg. per 24 ml. [b] 30° water bath, U.S.P. 95% ethanol, and 1:20 base dilution used.

TABLE VI.—EFFECT OF NITROGEN FLUSHING PRIOR TO COLOR DEVELOPMENT[a]

Experiment	Absorbance	
	Standard	Tablet
A[b]	0.387	0.420
B[c]	0.399	0.402
C[d]	0.428	0.430

[a] Prednisolone concentration = 0.25 mg. per 24 ml. [b] Chloroform evaporated under nitrogen but standard and blank flasks were not nitrogen flushed. [c] Chloroform evaporated in air. Standard and blank flasks were not flushed with nitrogen. [d] Chloroform evaporated under nitrogen. Standard and blank flasks were also flushed with nitrogen.

TABLE VII.—EFFECT OF TIME ON COLOR STABILITY OF TABLET EXTRACT[a]

Time, min.	Absorbance	
	Standard	Tablet Extract
30	0.442	0.443
60	0.441	0.441
90	0.435	0.439

[a] Prednisone concentration = 1.0 mg. per tablet.

Effect of Temperature on Color Development.—The rate of color development increases with temperature. Some fluctuations in assay results were obtained when (room) temperature fluctuations were present. For this reason, it was found expedient to adopt a standardized bath temperature of 30° as recommended by Johnson, King, and Vickers (1).

Color Stability.—When the stated precautions were observed, the stability of the developed color was very good. Almost no drop in absorbance was found after 2 hours in the 30° water bath, as shown in Table V.

Tablet Extract Preparation.—It was found that improper evaporation of the aliquot of the chloro-

TABLE VIII.—ANALYSIS OF PREDNISOLONE TABLETS BY THE MODIFIED PROCEDURE[a]

	Absorbance											
	Analyst A						Analyst B					
Sample	Std. 1	Std. 2	Tab. 1		Tab. 2		Std. 1	Std. 2	Tab. 1		Tab. 2	
A	0.465	0.472	0.442	0.440	0.441	0.435	0.458	0.466	0.450	0.450	0.460	0.460
B	0.464	0.466	0.403	0.406	0.404	0.402	0.458	0.466	0.433	0.430	0.432	0.433
C	0.465	0.465	0.422	0.423	0.454	0.458	0.467	0.468	0.430	0.432	0.452	0.452
D	0.462	0.462	0.452	0.450	0.470	0.469	0.467	0.468	0.452	0.454	0.462	0.464
E	0.465	0.465	0.450	0.462	0.440	0.434	0.458	0.466	0.448	0.453	0.456	0.456

[a] Steroid concentration = 5 mg. per tablet.

form extract of the tablet led to variable results if the higher boiling residues in the chloroform were not removed. This was easily effected by further heating the reaction flasks at about 95° under a stream of nitrogen for about 3 minutes longer after the flasks appeared to be dry. The flasks in which the standard and reagent blank were run also had to be flushed with nitrogen while still empty. This was found to be necessary because a comparison of the absorption readings of the solutions (obtained from the flasks nitrogen flushed and not nitrogen flushed at the start) indicated that higher absorbance values were obtained for the flushed flasks, as shown in Table VI.

It was also found that nitrogen flushing all the flasks while empty (as in the cases where chloroform was evaporated, just after the 3-minute extra heating time but before the addition of the alcohol) and nitrogen flushing again after all the reagents had been added was just as effective as the Johnson, King, and Vickers procedure of nitrogen flushing after each addition of reagent.

Tablet Extract Color Stability.—Table VII shows the rate of color development for prednisone extracted from tablets compared to standard prednisone. No substantial difference was found when ethanol U.S.P., 0.5% tetramethylammonium hydroxide concentration, and a 30° bath were used.

Tablet Analysis.—The assays performed on batches A through E whose results by the U.S.P. XVI procedure are shown in Table I were repeated using the new modified procedure. Aliquots of tablet powder from the same crushed tablets were used. Table VIII shows the results obtained by the two analysts on the five batches of tablets assayed on 5 consecutive days. The poor reproducibility previously noted on the standards and the day to day variation have practically disappeared.

Precision.—A comparison of the precision of the analyses between the U.S.P. XVI method (Table I) and the modified method (Table VIII) using the variances generated by the replicates indicates that the modified method is significantly better than the U.S.P. XVI method as indicated below.

Precision, calculated as σ (from replicates) $\times$ 100/(mean absorbance)

Sample	Method	Precision, %
Standards	U.S.P. XVI	2.3
	Modified	0.8
Tablets	U.S.P. XVI	1.7
	Modified	0.6

Applicability to Other Steroids.—Table IX indicates the rate of color development for steroids having modified environment in the side chain.

TABLE IX.—RATE OF COLOR DEVELOPMENT FOR VARIOUS STEROIDS[a]

	Molar Absorbance		
Steroid	1 Hour	2 Hours	3 Hours
Prednisone	16,600	16,900	16,800
Prednisolone	16,850	16,800	16,900
9α-Fluoro-16α-methyl prednisolone	17,150	17,450	17,100
9α-Fluoro-16β-methyl prednisolone	11,475	15,250	16,450
Desoxycorticosterone	16,500	16,300	16,200
Prednisone-21-acetate	17,000	17,300	17,400
Prednisone-21-hemisuccinate	13,100	15,100	15,150
9α-Fluoro-16α-methyl prednisolone phosphate	0	0	0
9α-Fluoro-16α-methyl prednisolone sulfate	0	0	0

[a] 30° water bath, U.S.P. 95% ethanol, and 1:20 base dilution used.

The color development fails completely when the 21-hydroxyl is firmly bound as in a phosphate or sulfate ester.

SUMMARY

The procedure reported by Johnson, King, and Vickers for bulk hydrocortisone has been extended to the assay of prednisone and prednisolone in tablets. The conditions found necessary to obtain satisfactory reproducibility are use of low actinic glassware, complete evaporation of the chloroform extracts under nitrogen, use of smaller tetramethylammonium hydroxide concentration and its addition last, flushing the empty part of the flasks containing the reagent solution with nitrogen, development of the color in a 30° water bath, and the use of ethanol with controlled water content such as U.S.P. 95% ethanol. When these conditions are fulfilled, the color shows no deterioration even after 2 hours at 30° in a closed flask.

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