Comparison of Triphenyltetrazolium and Blue Tetrazolium for Determination of Prednisolone

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Blue tetrazolium (3,3'-dianisole bis[4,4'-(3,5-diphenyl)tetrazolium chloride]) and 2,3,5-triphenyltetrazolium chloride are compared as colorimetric reagents for the assay of prednisolone. The effect of oxygen, light, temperature, and base concentration on the rate of color development and the stability of the blue tetrazolium reaction were investigated and found to be negligible. The procedure is applicable to other adrenocortical steroids.

A NUMBER of investigators have reported that the Mader and Buck (1) triphenyltetrazolium procedure for the determination of adrenocortical steroids described by the U.S.P. XVI gave poor reproducibility even though precise timing of the reaction was observed and meticulous care was taken in the cleaning of glassware. Johnson *et al.* (2) demonstrated the effect of oxygen, light, and base concentration on both the intensity of color and the rate of its formation with pure hydrocortisone, hydrocortisone acetate, prednisolone, and fludrocortisone; they recommended a procedure to control variance due to these factors. Callahan *et al.* (3) extended this study and applied the procedure to tablet formulations. They concluded, "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." While we found that Callahan's procedure would give reproducible results, it was hoped that a simpler method of equally acceptable accuracy could be developed for the routine analyses of pharmaceuticals.

The use of blue tetrazolium as a colorimetric reagent for steroid determinations has been reported in the literature with varying degrees of success. A rapid and sensitive blue tetrazolium procedure was published by Rechnagel and Litteria (4). Reproducible results were obtained when these precautions were observed: recrystallization of the blue tetrazolium from ether, the use of twice redistilled ethanol, the substitution of ethanolic sodium hydroxide solution for tetramethylammonium hydroxide solution, and restriction of the water content of the reaction mixture using anhydrous ethanol. In their study of those factors which influence the triphenyltetrazolium reagent, Johnson *et al.* (2) also compared that reagent with blue tetrazolium. They concluded that the advantages of greater sensitivity and freedom from interference by oxygen of the blue tetrazolium reagent are more than outweighed by the disadvantages of high and continuously varying blank readings. Smith and Halwer (5) used blue tetrazolium as one means of characterizing triamcinolone and the related 16 alpha hydroxy steroids. Because of the instability of the blue tetrazolium solution, they recommended that the reagent be prepared fresh daily.

Johnson and Francois (6) reported the successful use of Dajac B.T. brand blue tetrazolium without further purification for steroid studies. We investigated this particular reagent for its suitability in routine analyses. The reagent proved to be both sensitive and stable; a solution stored for 3 months in the dark showed no change in color development. The absorption of colors was proportional to concentration of the steroid in the 50–450 mcg. range examined. The maximum deviation was 0.7% from the mean in 126 analyses of U.S.P. prednisolone reference standard made over a 5-week period.

EXPERIMENTAL

Reagents

We used the following reagents: alcohol U.S.P., 95%; blue tetrazolium solution–dissolve 50 mg. of blue tetrazolium[1] in 10 ml. of alcohol by warming in a hot water bath and swirling; cool, filter, and protect solution from light. Dilute tetramethylammonium hydroxide—dilute 1 ml. of U.S.P. test solution to 20 ml. with alcohol.

Assay

Standard Preparation.—Using an accurately weighed amount of U.S.P. reference standard corresponding to the steroid under study, prepare an alcohol solution containing about 10 mcg./ml.

Sample Preparation.—For bulk steroids, prepare as directed under *Standard Preparation*. Transfer 20.0 ml. of the solution to a glass-stoppered 50-ml. conical flask for color development. For tablet preparations, weigh and finely powder not less than 10 tablets. Weigh accurately a portion of powder equivalent to about 5 mg. of the active ingredient and transfer it to a 125-ml. separator with the aid of 15 ml. of water. Extract the steroid using four 25-ml. portions of chloroform, filtering each portion through chloroform-washed glass wool into a 250-ml. volumetric flask. Add chloroform to volume and mix. Pipet 10 ml. of this solution into a glass-stoppered 50-ml. conical flask, evaporate the solution to dryness on a steam bath with a gentle current of air, cool, and dissolve the residue in 20.0 ml. of alcohol.

Color Development.—Into separate glass-stoppered 50-ml. conical flasks, pipet 20.0 ml. of the *Standard Preparation* and 20.0 ml. of alcohol to serve as a blank. To these flasks and to the flask containing the *Sample Preparation*, add 2.0 ml. of blue tetrazolium solution and mix. Add 2.0 ml. of dilute tetramethylammonium hydroxide and mix. Allow the solutions to stand in the dark for

Received December 10, 1963, from the Food and Drug Administration, Bureau of Scientific Research, Division of Pharmaceutical Chemistry, U. S. Department of Health, Education, and Welfare, Washington, D. C.
Accepted for publication February 26, 1964.

[1] Dajac, B. T., the Borden Co., Philadelphia, Pa., has been found satisfactory and was used for these studies.

90 minutes and, with a suitable spectrophotometer, determine the absorbances of the sample and standard solutions at the point of maximum absorbance at about 525 mμ relative to the reagent blank.

DISCUSSION

Melting point specifications and infrared and ultraviolet spectra do not distinguish between lots of blue tetrazolium suitable for steroid assay and those yielding a high blank and low color development. A simple suitability test is required prior to use of a new lot of this reagent. The absorbance of the colors developed must be proportional to the concentration of steroid; when assayed as directed under *Color Development*, the absorbance of the 200-mcg. level of hydrocortisone, corrected for the reagent blanks, should be no less than 0.500. Examination of nine lots of blue tetrazolium obtained from four different distributors indicates that such a test excludes unsuitable blue tetrazolium and yet allows considerable latitude for lot to lot variation. A high blank *per se* (above 0.100) does not necessarily preclude use of a particular lot of reagent. Of nine lots examined, two had absorbance blank values of 0.111 and 0.127, four were between 0.030 and 0.060, and three were unsuitable.

Those factors which have been reported to affect the intensity and stability of colors obtained with triphenyltetrazolium salts were studied and applied to the blue tetrazolium procedure. A series of tests using prednisolone showed that flushing the flasks with nitrogen prior to color development increased the color intensity by about 2%. Since reproducible absorption values were obtained without such nitrogen treatment, this precautionary step was eliminated. The use of a 1:10 dilution of tetramethylammonium hydroxide instead of the 1:20 dilution specified in the method gave no significant change in the absorbance values. Within the time interval examined, the order of the addition of the base and the color reagent has no effect on these values. Variations in room temperature between 22° and 30° did not affect reproducibility of the results. The use of actinic glassware showed no advantage over the use of clear glass when the solutions in the latter were protected from sunlight and incandescent light. Exposure to sunlight greatly increased the values obtained for the blanks when clear glass was used. In view of these observations, we recommend that direct sunlight be excluded from the laboratory when the reagents are added to the solutions and when the spectrophotometric measurements are being made. While the color developed with prednisolone and blue tetrazolium continued to increase in intensity over 18 hours, the rate of color development had decreased after 90 minutes to a point where it had negligible effect on the results.

RESULTS

Comparison of the U.S.P. XVI method modifications A and B represents work completed in this laboratory as a portion of a collaborative study designed and conducted by The Upjohn Co. Modification A reverses the order of the addition of triphenyltetrazolium and tetramethylammonium hydroxide. Modification B incorporates the precautions recommended by Callahan. The blue tetrazolium procedure was added to the study by the authors so that all three techniques could be studied under similar conditions. In each analysis 250 mcg. of prednisolone was used. The concentrations used were too high for optimum absorbance values for the blue tetrazolium reaction; however, it was felt that comparison would be simplified and additional variables avoided if equal aliquots of the same solutions were used throughout. The three methods were compared on three different days by two analysts. Each analyst prepared separate standards and weighed and extracted two samples of ground tablet formulation on each test day. Colors were developed in duplicate.

TABLE I.—RECOVERY OF PREDNISOLONE FROM TABLETS IN PER CENT OF LABEL CLAIM

	U.S.P. XVI Method Modification A	U.S.P. XVI Method Modification B	Blue Tetrazolium Procedure
Analyst A			
Day 1	100.5	96.7	99.6
	99.7	96.0	99.5
Day 2	100.3	99.6	100.4
	100.3	99.6	100.5
Day 3	101.1	99.6	101.1
	100.4	98.7	100.9
Analyst B			
Day 1	98.7	100.9	100.5
	96.6	102.4	100.1
Day 2	99.5	101.0	101.2
	98.7	99.8	101.2
Day 3	100.3	102.9	101.3
	100.0	102.2	101.1
Max. range	96.6 to 101.1	96.0 to 102.9	99.5 to 101.3

The results of this study are presented in Table I. While the results by all three procedures showed fair reproducibility, those obtained by the blue tetrazolium method were by far the most consistent. This fact and the ease with which this procedure can be applied would make it appear to be the method of choice.

The blue tetrazolium reaction is applicable to other adrenocortical steroids. It was used to quantitate those fractions isolated by paper chromatography in the procedure "Determination of the Major Component of Some U.S.P. Adrenocortical Steroids" which is presented in a separate paper (7).

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