Stabilization of Tetrazolium Blue Assay for Triamcinolone

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The substitution of chloroform to the extent of 60 per cent for the pure ethanol normally used in performing the tetrazolium blue assay of triamcinolone produces a stable color in both the sample solution and the reagent blank. Stable color development is achieved within 20 minutes for triamcinolone and its acetonide and diacetate derivatives. The reagent blank reaches a constant value within 10 minutes. In addition, stabilization of the tetrazolium blue reaction overcomes the major objection to its general use and results in a more favorable comparison with triphenyl tetrazolium as the reagent of choice. While the work reported is confined to triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20 dione) and two of its derivatives, it appears reasonable to assume that the technique described could be extended to the assay of other ketol steroids.

THE APPLICATION of tetrazolium salts for the determination of ketol steroids was presented by Mader and Buck (1). Smith and Halwer (2) applied this reaction to the determination of triamcinolone alcohol, acetonide, and diacetate. Tetrazolium blue was the specific reagent used in this work. Johnson, King, and Vickers (3) reported that the use of tetrazolium blue as an analytical reagent is complicated by the fact that neither the reagent blank nor the sample achieve a stable color within a reasonable time. The problem was regarded as serious enough by these authors to state a general preference for triphenyl tetrazolium since their observations indicated that this compound did not have this undesirable characteristic. However, tetrazolium blue does show better resistance to attack by oxygen (3) and is approximately twice as sensitive a reagent as triphenyl tetrazolium (1). The work described below allows one to take advantage of the desirable features of tetrazolium blue by eliminating the continuous increase in absorbance of the sample and reagent blank.

DISCUSSION

Origin of Investigation.—The use of chloroform to stabilize the blue-colored formazan produced during the reduction of tetrazolium blue by triamcinolone originated as a result of the application of this solvent to the extraction of triamcinolone acetonide from ointment preparations. The use of chloroform for such extractions is not uncommon (4, 5). However, the chloroform is removed by evaporation prior to assay. While attempting to reduce assay time by eliminating this step, it became obvious that not only could the color reaction be carried out in the presence of chloroform, but also the resulting solutions exhibited better stability than when the chloroform was absent. Subsequent work was carried out to investigate systematically this observation.

Effect of Chloroform on Color Development.—Samples of triamcinolone alcohol, acetonide, and diacetate were dissolved in ethanol. Equal aliquots were transferred to a series of flasks for each of the steroids tested. Varying amounts of chloroform were then added to each sequence and the tetrazolium blue assay completed. Table I gives the data obtained. After examination of these data, it was concluded that a 60% chloroform content represented the concentration of chloroform that would yield the maximum color response consistent with the primary purpose of stabilizing the reaction.

Rate of Color Development in 60% Chloroform.—Assays were performed using the 60% chloroform level to determine the amount of time needed for each of the compounds in question and for the reagent blank to reach a plateau in their absorbance values. An identical set of assays was performed in ethanol alone to provide comparative data between the two solvents. Table II summarizes the results obtained.

It was concluded that the absorbance values obtained between 20 and 30 minutes (after color development was initiated in the chloroform–ethanol mixture) could safely be regarded as having achieved a stable maximum value. This should conservatively estimate the span of time suitable for accurate quantitation. For example, data in Table I indicate at least 1 hour of color stability when triamcinolone alcohol is being tested, and 2 hours' stability for the reagent blank. In addition, 20 minutes is more than sufficient time for maximum color development except for triamcinolone alcohol as shown in Table II.

Linearity of Response.—Standard calibration curves were prepared for all three steroids tested. Typical Beer's law plots were obtained over the concentration range of 1 mcg./ml. to 5 mcg./ml. for all three compounds. The data are presented in Fig. 1.

Effect of Light.—Sensitivity to light remains a problem in the performance of this assay. The use of low actinic glassware as noted by Johnson, *et al.* (3), or storage in the dark until the absorbance values are to be determined, is necessary.

EXPERIMENTAL

Assay Reagents.—(a) Tetramethylammonium hydroxide, supplied as a 10% aqueous solution. Prepare a working solution by diluting 10 ml. to 50 ml. with 95% ethanol; (b) Tetrazolium blue, dissolve 350 mg. in 100 ml. of 95% ethanol; (c) 95% ethanol, essentially aldehyde-free; (d) chloroform, reagent grade.

Assay Procedures.—Prepare a chloroform solution of the steroid to be assayed using a concentration of 5 mcg./ml. for triamcinolone alcohol[1] and diacetate or 10 mcg./ml. for the acetonide. Transfer 15 ml. of the chloroform solution to a 25-ml. low actinic volumetric flask. At the same time prepare a standard of the steroid being assayed to carry through the test. A reagent blank is also prepared by adding 15

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[1] Triamcinolone alcohol is more easily dissolved in ethanol than chloroform. Initial solution should be made in ethanol and a second dilution at 5 mcg./ml. in chloroform prepared

TABLE I.—ABSORBANCE VALUES AT 520 mμ SHOWING THE EFFECT OF THE CHLOROFORM-TO-ETHANOL RATIO ON COLOR DEVELOPMENT[a]

Chloroform, %	Triamcinolone Alcohol, Reaction Time, Min. 20	Triamcinolone Alcohol, Reaction Time, Min. 60	Triamcinolone Acetonide, Reaction Time, Min. 20	Triamcinolone Diacetate, Reaction Time, Min. 20	Reagent Blank, Reaction Time, Min. 20	Reagent Blank, Reaction Time, Min. 60	Reagent Blank, Reaction Time, Min. 120
0	0.328	0.461	0.243	0.224	0.061	0.086	0.102
10	0.408	...	0.310	0.345	0.063	0.081	...
20	0.464	0.513	0.350	0.390	0.070	0.083	0.099
40	0.498	0.513	0.395	0.408	0.081	0.084	0.084
60	0.482	0.483	0.392	0.394	0.084	0.084	0.084
70	0.460	...	0.380	0.361	0.084	0.084	...
80	0.418	0.418	0.361	0.330	0.084	0.084	0.084

[a] The concentrations of the three compounds tested were 0.003, 0.006, and 0.003 mg./ml. for triamcinolone alcohol, acetonide, and diacetate, respectively.

TABLE II.—ABSORBANCE VALUES AT 520 mμ SHOWING THE RATE AND STABILITY OF COLOR DEVELOPMENT IN 60% CHLOROFORM-ETHANOL *vs.* ETHANOL ALONE[a]

Time, min.	Triamcinolone Alcohol, Et.OH	Triamcinolone Alcohol, Et.OH/$CHCl_3$	Triamcinolone Acetonide, Et.OH	Triamcinolone Acetonide, Et.OH/$CHCl_3$	Triamcinolone Diacetate, Et.OH	Triamcinolone Diacetate, Et.OH/$CHCl_3$	Reagent Blank, Et.OH	Reagent Blank, Et.OH/$CHCl_3$
5	0.320	0.730	0.230	0.560	0.280	0.535	...	0.078
10	0.505	0.785	0.390	0.575	0.450	0.562	...	0.085
15	0.605	0.792	0.488	0.575	0.535	0.565	0.091	0.085
20	0.665	0.795	0.540	0.575	0.585	0.565	0.176	0.085
25	0.700	0.795	0.565	0.575	0.610	0.565	0.210	0.085
30	0.730	0.797	0.580	0.575	0.635	0.565	0.268	0.085

[a] The concentrations of the three compounds tested were 0.006, 0.012, and 0.006 mg./ml. for triamcinolone alcohol, acetonide, and diacetate, respectively.

ml. of chloroform to a third 25-ml. volumetric flask. All three flasks are taken through the following procedure.

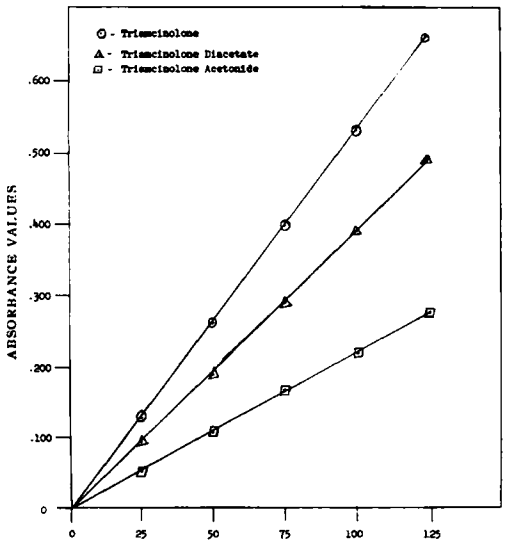

Fig. 1.—Standard calibration curves for triamcinolone, triamcinolone diacetate, and triamcinolone acetonide.

Add 1 ml. of tetrazolium blue solution to each of the flasks, followed immediately by 1 ml. of the tetramethylammonium hydroxide working solution. Bring all flasks to volume with 95% ethanol. Mix well and allow the flasks to stand 20 minutes. Determine the absorbance values for each of the solutions at 520 mμ in any suitable spectrophotometer using ethanol as the reference solution. The value of the steroid content of the unknown samples may then be calculated by comparison with the standard after the reagent blank is subtracted from both.

APPLICATION OF PROCEDURE

This procedure has been applied to routine determination of the steroidal content of a wide variety of pharmaceutical formulations of all three steroids. Tablets, creams, ointments, and suspensions have been successfully assayed using this method. The chloroform often serves a dual purpose by assisting in extracting the steroid from the formulation as well as stabilizing the color reaction.

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