Differential Borohydride Assay for Conjugated Ketosteroids: Improvement with Propylene Glycol

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Abstract □ A previously developed differential UV assay for 3-one-4-ene steroids involving reduction with sodium borohydride was frequently found to be less than 100% complete. Such incomplete reduction can be mimicked by additions of sodium metaborate, a product of the hydrolysis of sodium borohydride, to the reaction system. Complete reduction can be achieved by adding propylene glycol to the reduction system. The phenomenon was studied using halcinonide as a model.

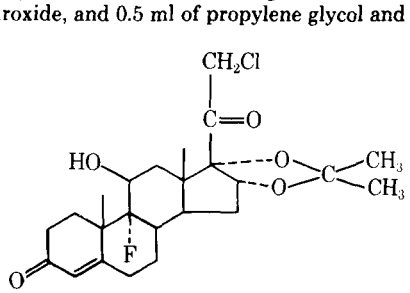
Keyphrases □ Steroids, 3-one-4-ene—effect of added propylene glycol on differential UV analysis □ UV spectrophotometry, differential—analysis of various 3-one-4-ene steroids, effect of added propylene glycol □ Propylene glycol—effect of addition on differential UV analysis of various 3-one-4-ene steroids

While developing a differential UV assay for the 3-one-4-ene steroid halcinonide[1] (I), a topical anti-inflammatory agent, sodium borohydride reagent frequently gave incomplete reduction of the steroid. The method was adapted from the procedure of Görög (1), as modified by Chafetz *et al.* (2), for quantitating conjugated 3-ketosteroids. This differential assay involves measuring the absorbance of an aliquot of methanolic steroid solution containing sodium borohydride, decomposed prior to the addition of steroid. Its absorbance is determined against a methanolic reference solution of steroid reduced by sodium borohydride. This simple procedure eliminates many interferences from excipients and other unconjugated steroids that may be present in the sample.

Sodium borohydride solutions prepared from some bottles of the reducing agent gave incomplete reduction of halcinonide. This paper reports that such incomplete reductions can be mimicked by the addition of sodium metaborate ($NaBO_2$) to the reaction system. Sodium metaborate is a known product of the hydrolysis of sodium borohydride (3). Complete reduction can be obtained when glycols, especially propylene glycol, are present in the reaction mixture.

EXPERIMENTAL

Reagents—Reagent grade anhydrous methanol was used. Sodium borohydride–propylene glycol reagent was prepared by adding 460 mg of sodium borohydride to a flask containing 8 ml of methanol, 2 ml of 1 *N* sodium hydroxide, and 0.5 ml of propylene glycol and mixing. This reagent was used not less than 45 min after preparation and was prepared fresh daily.



I

Methanolic 1 *N* HCl was prepared by diluting 8.5 ml of concentrated acid to 100 ml with methanol. To prepare the halcinonide standard, approximately 25 mg of steroid was dissolved in 100 ml of methanol, with the aid of an ultrasonic bath when necessary. This solution was diluted fivefold with methanol.

Differential Assay—Pipet 5.0-ml aliquots of standard or assay solution into the bottom of 25-ml volumetric flasks, designated standard blank or sample blank, followed by 1.0 ml of the sodium borohydride reagent. Wash down the sides of the flasks with about 1 ml of methanol and then gently swirl the flasks to mix the contents. After permitting the flasks to stand for 30–35 min with occasional swirling, destroy the excess borohydride by adding 2.0 ml of 1 *N* methanolic hydrochloric acid. Place the flasks briefly in an ultrasonic bath to hasten the removal of dissolved gases, dilute to volume with methanol, and then mix.

Pipet 1.0-ml aliquots of the same sodium borohydride reagent solution into the bottom of 25-ml volumetric flasks, designated sample or standard, followed by 2.0 ml of methanolic hydrochloric acid. Wash down the sides and rims with methanol and then swirl the flasks. After about 15 min, pipet 5.0 ml of sample or standard solution into its respective flask, dilute to volume with methanol, and mix.

Determine the absorbance of each sample or standard against its respective reduced reference blank at 350 nm and at the peak maximum at about 239 nm, using matched 1-cm cells and a suitable spectrophotometer. If the absorbance at 350 nm exceeds ±0.003, wash the outside of the cells with methanol to remove any contamination with the sodium borohydride–hydrochloric acid reaction mixture. Use the algebraic difference between absorbances at 239 and 350 nm to calculate the halcinonide content of the sample based on the concentration and absorbances of the standard. Formulations of halcinonide can be extracted or diluted with methanol prior to the assay.

TLC—The amount of sodium metaborate in various lots of sodium borohydride was estimated by a TLC procedure (4). Plates[2], 250 μm thick, were developed with 0.1 *N* aqueous hydrochloric acid and dried for 2 hr at 125°. Samples of 25 μl of borohydride (prepared by following the recommended procedure), standards (1, 2, or 3% in methanolic sodium hydroxide; the last concentration spotted one to four times), and blank (methanolic sodium hydroxide) were spotted on several plates with 25-μl syringes[3]. The developing solvent was methanol–1-butanol–water (3:1:1). After development and warm air drying, the plates were sprayed with stabilized starch solution[4], redried, and then sprayed with 0.1 *N* iodine in potassium iodide (5).

RESULTS AND DISCUSSION

Sodium borohydride reduces the carbonyl function in the 3-one-4-ene steroid A ring of halcinonide to a hydroxyl group, thus destroying the chromophore and eliminating steroid UV peak absorbance. The extent of reduction is determined by comparing the absorbance of an aliquot of steroid diluted with methanol with the absorbance of a similarly diluted aliquot of differentially reduced steroid (1). Incomplete reduction was often found with various bottles of sodium borohydride from different lots.

Since sodium borohydride is readily hydrolyzed to sodium metaborate (Scheme I) (3), the simplest working hypothesis is that sodium metaborate, or a compound related to it, replaces or affects the active borohydride.

$$NaBH_4 + 2H_2O \rightarrow NaBO_2 + 4H_2$$

Scheme I

[1] Halog Cream, E. R. Squibb and Sons.

[2] Mallinckrodt Chromar.

[3] Hamilton.

[4] Fisher.

Experimentally, adding metaborate to sodium borohydride reagent or replacing borohydride with metaborate results in diminished reduction. Since sufficient borohydride is present in control experiments to give complete reduction, this incomplete or inhibited reduction may be caused by metaborate (or a related compound), perhaps *via* complexation with the enol form of the 3-one.

Evidence for decomposition of the borohydride at the surface comes from iodometric titrations (6) of sodium borohydride solutions. Material taken from the surface of three bottles of borohydride showed 1% less borohydride content by titration than material taken from the bottom of the bottle. When used in the differential assay, such material removed from the surface also gave 1–4% less halcinonide reduction than did material from the bottom of the bottles. Sodium metaborate itself may not be responsible for the inhibition of reduction; such a compound as sodium borinate ($NaBH_2O$) may be the inhibitor in borohydride solutions.

As determined by TLC, the content of metaborate (R_f 0.11) was equal to or less than 1%, as estimated by visual comparison with standards, for borohydride giving 99% reduction. Borohydride appeared to remain near the origin (R_f 0.02). Light spots were visible against a dark background. Approximately 2% metaborate was found in borohydride solution giving 97% reduction, and ~10% metaborate was found in borohydride reducing 82% of the halcinonide present. This final value may be erroneously low because of the presence of an insoluble suspension in the borohydride solution. The sodium metaborate content of these three lots of sodium borohydride was inversely related to their reducing ability.

Glycols and other compounds containing vicinal hydroxyl groups sequester borate ions (7). Mannitol or glucose added to halcinonide gave increased differential absorbances, indicative of greater reduction. Glycerol additions permitted 100% reduction over 30–35 min. However, propylene glycol gave 100% reduction over a wider range of conditions and did not contribute to the absorbance of the differential reduction (*cf.*, discussion of variables below).

Propylene glycol blocks inhibition of reduction by sodium metaborate, as was seen when propylene glycol was added to halcinonide standard. Reduction of 100% was found with 0–5% sodium metaborate added to sodium borohydride solution containing propylene glycol, 99% reduction was found with 10% added metaborate, and 95% reduction resulted with 15% added metaborate.

Sodium borohydride, supplied by three vendors, gave reductions that were 92–98% complete when powder from the surface was used. Following the recommended addition of propylene glycol, reductions were 100 ± 0.5% complete. Borohydride that was solidified to the extent that scraping the surface dislodged only a few milligrams at a time gave 82% reduction without added propylene glycol and 88% with the recommended quantity. Such solidified, rock-like borohydride should be discarded.

The recommended molar ratio of sodium borohydride to halcinonide is approximately 2100:1. In spite of the large excess, an iodometric assay (6) showed that the quantity of sodium borohydride remaining after the 30-min reduction of halcinonide solution was similar, within a 1% experimental error, to the borohydride content of solutions unreacted with borohydride. This result indicates that the excess borohydride was not decomposed and was necessary for quantitative reduction.

Table I shows that other 3-one-4-ene and 3-one-4,6-diene steroids are reduced to the same extent as halcinonide with the recommended procedure. 3-One-1,4-diene steroids are less than 10% reduced, limiting this procedure. The procedure of Chafetz *et al.* (2), using lithium borohydride, also reduces 1,4-diene steroids. Two samples of 3–5-year-old lithium borohydride gave incomplete reductions of halcinonide and triamcinolone after the recommended procedure was modified for equimolar lithium borohydride. The addition of propylene glycol increased the extent of reduction. Such use of propylene glycol could be of general utility in increasing yields during large-scale reductions with borohydrides. The concentration of glycol may have to be adjusted for each reaction, since too much propylene glycol inhibits reduction, as shown in the following discussion of variables.

Complete reduction, using either propylene glycol added to solutions of halcinonide standard or to borohydride reduction reagent, was found between 25 and 45 min. The reaction is judged complete when the absorbance of aliquots of halcinonide standard carried through the recommended procedure is equal to the differential absorbance of an aliquot of halcinonide diluted in methanol and read *versus* a methanol blank. Recoveries averaged 100.1% (n = 11), with a coefficient of variation of 0.42.

Table I—Differential Sodium Borohydride Reduction of Other 3-One-4-ene Steroids

Name	Structural Feature	Without Propylene Glycol, %	With Propylene Glycol, %
Hydrocortisone	3-One-4-ene	93	100
Progesterone	3-One-4-ene	98	100
Fluoxymesterone	3-One-4-ene	96	100
Triamcinolone	3-One-1,4-diene	2	3
Dexamethasone	3-One-1,4-diene	3	3
Betamethasone	3-One-1,4-diene	3	3
Prednisolone	3-One-1,4-diene	6	6
Chlormadinone	3-One-4,6-diene	99	100

Reductions without propylene glycol added to standards were 94–98% complete, depending on the lot of borohydride used. Using 1.0–4.0 ml of propylene glycol/50 ml of standard solution yielded 100% reduction within 25 of the recommended 30 min. Five milliliters of propylene glycol gave 95–99% reduction, and 10 ml gave 92% reduction. Several different grades of purity of propylene glycol, including glass distilled, were used and gave identical results. Three milliliters of propylene glycol in the absence of halcinonide had negligible absorbance.

Complete reduction of halcinonide was obtained using methanolic sodium hydroxide solution, with final sodium hydroxide normalities ranging from 0.30 to 0.02 N, which provides a buffer against possible contaminating acid.

Volumes of sodium borohydride reagent (from a lot that appeared to be free of metaborate) between 0.7 ml (31 mg) and 2.0 ml (88 mg) gave 100 ± 0.5% reduction. The percentage of halcinonide reduced with the recommended 1.0 ml of sodium borohydride reagent ranged between 99 and 101% in all studies. Below 0.6 ml, reduction was incomplete; concentrations above 2.0 ml were not investigated.

The procedures described here give results that are within 1% of anticipated steroid content in contrast to the previous, generally low-biased results.

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