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N-Bromosuccinimide Assay of Penicillins and Cephalosporins

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Abstract □ All penicillins and cephalosporins known to possess biological activity respond to an *N*-bromosuccinimide assay. The developed method is not yet usable for determining stability, but it is useful as a bulk or batching assay.

Keyphrases □ *N*-Bromosuccinimide—reagent for iodometric analysis of penicillins and cephalosporins □ Penicillins—analysis, using *N*-bromosuccinimide, iodometric titration □ Cephalosporins—analysis, using *N*-bromosuccinimide, iodometric titration □ Titrimetry, iodometric—analysis of penicillins and cephalosporins

The iodometric assay for penicillin has been used successfully for almost 30 years (1), and it also has been used for cephalosporins (2). Recently, the results of some iodometric assays of synthetic cephalosporins proved difficult to interpret because of the complexity of the side chains. For such cases, another simple and rapid method was sought that might permit easier interpretation of the assays of complicated structures and that might supplement the iodometric assay.

A bromometric method, reported for penicillin O (3), differed from the iodometric method in that no inactivation of penicillin with alkali was required prior to the measurement. In this laboratory, the reaction of *N*-bromosuccinimide with penicillin was found to require no inactivation of the antibiotic with alkali or penicillinase. An attempt to use a direct assay procedure with this reagent, which had been used for several years by a host of investigators (4–12), led to the realization that the reaction between the penicillins and *N*-bromosuccinimide was time dependent. Moreover, the reagent was found to be somewhat light sensitive and subject to oxidation in air when used as a direct titrant for penicillins, in spite of its prior use in the determination of such simple compounds as sulfides, thiocyanates, cysteine, thiourea, mercaptans, phenols, and sulfur-containing amino acids (4–12). Technical grade *N*-bromosuccinimide deteriorated rapidly in solution.

To offset these difficulties, a different approach was used. In experiments involving the reaction of penicillin with an excess of *N*-bromosuccinimide, a rapid rise in the consumption of *N*-bromosuccinimide was evident from time zero to 1 hr, after which consumption increased at a slower rate. The most reproducible results were obtained when the reaction time was limited to 2.0 hr; it is suggested that this period be used. Results on samples shielded from light or assayed at a lower temperature did not differ significantly from those obtained under the described assay conditions.

EXPERIMENTAL

Reagents—*0.02* N *N-Bromosuccinimide*[1]—Dissolve 1.9 g of *N*-bromosuccinimide in about 5 ml of reagent grade dimethylformamide. Pour into a 1-liter volumetric flask containing about 500 ml of distilled water. Stir or shake and then dilute to volume with distilled water. Transfer to a dark, amber bottle and store immediately in the refrigerator.

Despite reports of the instability of *N*-bromosuccinimide, this solution will maintain its titer for several weeks under proper storage conditions, with a loss of less than 5% of its original value.

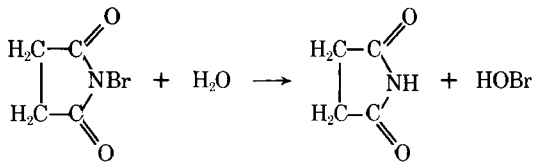
0.01 N *Sodium Thiosulfate*—Dissolve 2.5 g of sodium thiosulfate pentahydrate in cooled, freshly boiled distilled water and add 2–3 ml of toluene (preservative). Then dilute to 1 liter with water.

Buffer Solution—To 40 g of anhydrous sodium acetate dissolved in about 500 ml of distilled water, add 1.93 ml of concentrated sulfuric acid. Dilute to 1 liter with distilled water.

Starch Solution—Prepare a 1% solution of starch in saturated saline.

Dimethylformamide (reagent grade), potassium iodide (reagent grade), and acetic acid were used also.

[1] Aldrich analyzed reagent grade, Aldrich Co., Milwaukee, WI 53233



Scheme I

Standardization of *N*-Bromosuccinimide Reagent—Pipet exactly 5 ml of *N*-bromosuccinimide solution into a 125-ml flask. Add 5 ml of the buffer solution to simulate the conditions used in the actual analysis. Add 1 ml of acetic acid and then 100–200 mg of solid potassium iodide, and let the solution stand for 3–5 min. Titrate the liberated iodine with 0.01 *N* sodium thiosulfate solution, using starch when approaching the end-point (indicated by the paleness of the iodine color).

This *N*-bromosuccinimide solution is 0.2 *N* with respect to the thiosulfate solution and is actually 0.01 *M*. The *N*-bromosuccinimide solution is twice the strength of the thiosulfate solution (to maintain the excess needed for the assay) and should result in a titer of about 10 ml of sodium thiosulfate solution; the titer is determined *daily*. This value, called R_4, can be referred to as *the blank*.

Assay—Prepare an aqueous solution of the test sample so that it contains approximately 0.2–0.3 mg/ml. If the material to be determined is insoluble in water, dissolve it in a few milliliters of dimethylformamide and add water to attain the desired volume. Pipet two 5.0-ml aliquots into two 125-ml flasks, R_1 and R_2, and add 5 ml of buffer solution to each. To a third flask, R_3, add about the same amount of reference standard and buffer. The fourth flask, R_4, is the blank.

To each flask, add exactly 5.0 ml of the *N*-bromosuccinimide solution and set a timer for a preselected interval (usually 1 or 2 hr). At the end of this time, add 1 ml of acetic acid and 100–200 mg of potassium iodide. Then titrate the liberated iodine in each flask as directed in the standardization procedure.

Calculations—Since this method can be used for the assay of all penicillins and cephalosporins, the calculation factor (C.F.) will vary for each species to be determined. The generic formula is:

$$\frac{\text{ml of thiosulfate} \times \text{C.F.} \times 1000}{\text{mg of sample}} = \mu\text{g} \qquad \text{(Eq. 1)}$$

where:

ml of thiosulfate = difference in milliliters between the amount required for titration of the blank and of the sample

C.F. = the ratio of the weight of the reference standard for the particular compound to milliliters of thiosulfate, determined as expressed in Eq. 2:

$$\text{C.F.} = \frac{\text{mg of reference standard}}{\text{ml of 0.01 } N \text{ } Na_2S_2O_3 \text{ } (R_3)} \qquad \text{(Eq. 2)}$$

The complete calculation may, therefore, be represented as:

$$R_4 - \left(\frac{R_1 + R_2}{2}\right) \times \frac{\text{mg of standard}}{R_3} \times 1000 = \mu\text{g/mg} \qquad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

The penicillins assayed included penicillin G potassium, ampicillin, penicillin V, epicillin, cloxacillin, cloxacillin benzathine, and oxacillin. The cephalosporins assayed were cephradine, cephalexin, cephalothin, cephaloridine, cephaloglycin, and numerous cephalosporin candidates. All gave responses with *N*-bromosuccinimide. Notably, sulfoxides did not react. Essentially, the reaction (11) involves the formation of hypobromous acid (Scheme I).

The hypobromous acid attacks the ring system and, because of its great oxidizing capacity, yields a large number of equivalents of iodine. A study showed that 10–17 equivalents of iodine were consumed when cephradine was allowed to react with *N*-bromosuccinimide for 0.5–6 hr. Therefore, the reaction time must be controlled. More consistent results were obtained at about 2 hr when the reaction curve started to flatten. Experiments with cephradine indicated that approximately 14 equivalents of iodine were used for a reaction time of 2 hr. Because the reagents used in routine analysis of penicillins and cephalosporins are standardized against pure reference standards, that practice was continued here. Nevertheless, in experimental work, one can determine the number of equivalents of iodine from the following:

$$\frac{\text{mg of reference material}}{\text{ml of sodium thiosulfate} \times \text{normality}} = \text{equivalent wt.} \qquad \text{(Eq. 4)}$$

$$\frac{\text{mol. wt. of reference material}}{\text{equivalent wt.}} = \text{number of } I_2 \text{ equivalents} \qquad \text{(Eq. 5)}$$

Experiments on formulated products showed some interferences that would prevent the direct application of this method; some separation step must be included in the analysis of such products. On purified materials, the results corresponded to those obtained by the iodometric method, but the method, unlike the iodometric assay, does not provide an indication of stability. For samples of cephalosporins with a complicated structure, the results obtained by this method were in better agreement with the results of the biological assay than were those obtained by the iodometric assay.

In contrast to the iodometric method, which shows a twofold difference between cephalosporins and penicillins in uptake of iodine (2), the *N*-bromosuccinimide assay shows very little difference in this respect. This contrast prompts some speculation about the relative ease of oxidation of the two classes of compounds. As mentioned earlier, penicillanic acid, 7-aminodesacetylcephalosporanic acid, and penicillamine react with *N*-bromosuccinimide, but the sulfoxides of penicillins and cephalosporins do not.

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