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ACKNOWLEDGMENTS AND ADDRESSES

Received June 3, 1974, from the *Department of Pharmaceutical Sciences, School of Pharmacy, and the Department of Neurological Surgery, School of Medicine, University of Washington, Seattle, WA 98195*

Accepted for publication August 19, 1974.

Supported by Contract N01-NS-1-2282 and Grant NS04053 of the National Institute of Neurological Disease and Stroke.

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PHARMACEUTICAL ANALYSIS

New Color Reaction for Determination of Bacitracin in Ophthalmic Ointments

J. DOULAKAS

Abstract □ A colorimetric method was developed for the rapid analysis of bacitracin in ophthalmic ointments. The method involves the oxidation of α-aminocarboxylic acid with sodium hypobromite in an alkaline medium and condensation of the resulting aldehyde with phloroglucinol in concentrated hydrochloric acid to yield a pink color which gives an absorbance maximum at 505 nm. The relationship between absorbance and the quantity of bacitracin reacted obeyed Beer's law over the 15–100-μg/ml concentration range studied, and the produced color was stable for several hours. Furthermore, the method, which can be applied directly to the aqueous dissolution sample, gave results comparable to the official microbiological analytical procedure. The standard deviation is equal to ±1.81%.

Keyphrases □ Bacitracin—colorimetric analysis in ophthalmic ointments □ Ophthalmic ointments—colorimetric analysis of bacitracin □ Ointments, ophthalmic—colorimetric analysis of bacitracin □ Colorimetry—analysis, bacitracin in ophthalmic ointments

Although numerous microbiological methods (1–9) have been reported for the determination of bacitracin, the chemical analysis of this antibiotic in dosage forms has been a difficult task. The current widely accepted procedure for the determination of bacitracin employs a microbiological assay. In general, this procedure requires considerably more time than do chemical methods. For this reason, it was thought desirable to develop a chemical assay for the antibiotic in the hope of achieving rapid results with greater accuracy and precision. UV spectrophotometry, because of the very low absorbance of bacitracin, can be used for identification purposes rather than for a quantitative determination (10, 11).

Stretton *et al.* (12) reported the separation of zinc bacitracin, neomycin sulfate, and polymyxin B sulfate by various chromatographic procedures. They worked with the pure antibiotics and determined them colorimetrically, using a ninhydrin reagent, according to Maehr and Schaffner (13), after previous electrophoretic separation.

A simpler and shorter approach seemed to be one which might take advantage of the lability of the aminocarboxylic groups toward oxidation. Since these groups can be oxidized to the aldehyde (14) by sodium hypobromite fairly readily and further condensed with phloroglucinol (15), it was anticipated that this technique could be employed for a quantitative assay of bacitracin.

In the present investigation, it was found that bacitracin could be quantitatively determined by employing a colorimetric technique. The procedure is based upon heating the antibiotic with alkali sodium

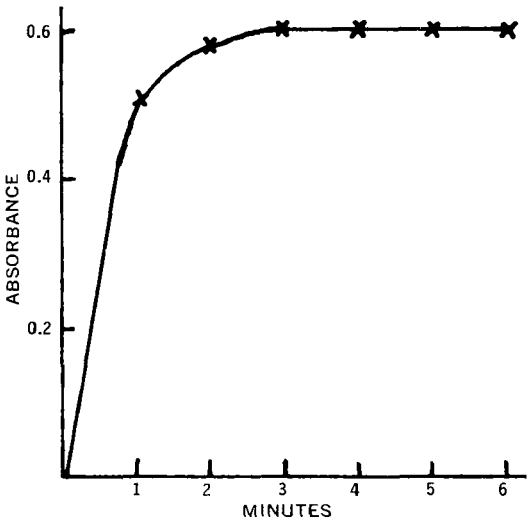


Figure 1—*Reaction time at 100°.*

hypobromite and condensing the resulting aldehyde with phloroglucinol reagent in an acid medium. The absorbance of the color produced is measured spectrophotometrically at 505 nm. The color-producing reaction with bacitracin gives excellent results and has not been previously reported.

EXPERIMENTAL

Instrumentation—Spectra and absorbance measurements were made with a double-beam spectrophotometer[1]. Matched glass-cells with a 2-cm optical path were used.

Reagents and Chemicals—Bromine and phloroglucinol, analytical reagent grade[2], were used as the chromogenic reagents. All solvents and other reagents used were also analytical reagent grade[2].

Sodium Hypobromite Solution—Dissolve 2 g of bromine into a 100-ml glass-stoppered volumetric flask, which has been previously half-filled with 1 *N* sodium hydroxide solution. Dilute to volume with the same solvent.

Phloroglucinol Reagent—Dissolve 0.5 g of phloroglucinol, in a 50-ml glass-stoppered volumetric flask, in 15 ml of distilled water by warming in hot water and shaking. Dilute to volume with concentrated hydrochloric acid (37%).

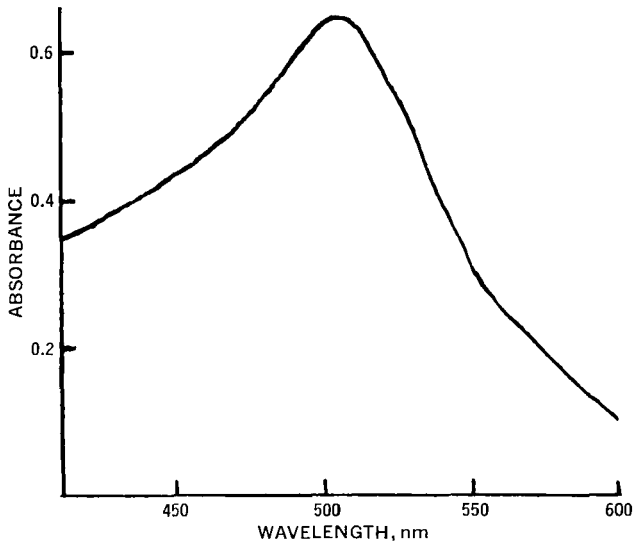


Figure 2—*Absorption spectrum of bacitracin (80 μg/ml).*

[1] Hitachi Perkin-Elmer model 124 (UV and visible).
[2] E. Merck Ltd., Darmstadt, West Germany.

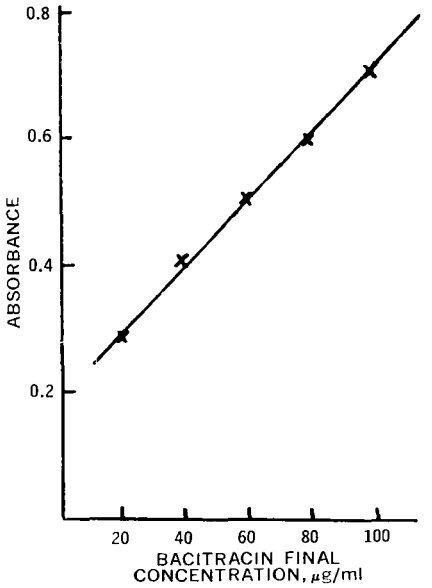


Figure 3—*Relationship between absorbance and concentration of bacitracin.*

Standard Reference Solution—Bacitracin[3], pure according to BP 1968, with a biological potency of 65 units/mg, was used. The purity of the commercially available bacitracin was checked by UV, TLC, and microbiological assay and was found acceptable. It was used as such without further purification. Bacitracin (10 mg) was dissolved in distilled water in a 50-ml glass-stoppered volumetric flask and diluted to volume with the same solvent.

Analytical Procedure—Weigh accurately a sample of the ophthalmic ointment, equivalent to approximately 10 mg (650 units) of bacitracin, into a 50-ml glass-stoppered centrifuge tube fitted with a ground-glass joint. Add 2 ml of ligroin (40–60°), warm slightly (*e.g.*, in a water bath at 40°), and shake briefly until the contents of the tube become a homogeneous viscous mass. Add 4.0 ml of distilled water and shake mechanically for 1 hr.

To assist solution of the active compounds, interrupt the shaking two or three times for 1-min intervals and place the tube briefly in the water bath each time. Finally, centrifuge for 5 min at 3000 rpm. Remove the aqueous (lower) phase with the help of a pipet, and filter it through a filter paper previously washed with distilled water and dried. Pipet 2.0 ml of the filtrate into a 25-ml glass-stoppered volumetric flask and fill to volume with distilled water.

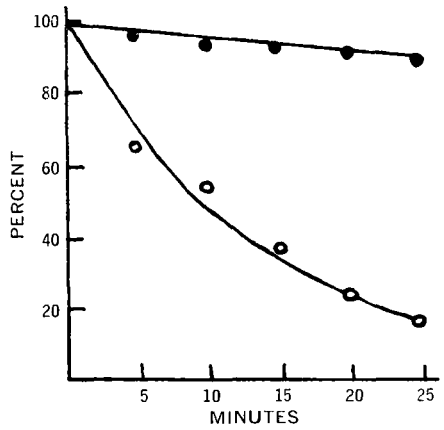


Figure 4—*Decomposition of bacitracin at 50 ± 0.1° (pH 13). Key: ●, colorimetric determination; and ○, biological determination (1 mg/ml).*

[3] Sigma Chemical Co.

Table I—Analysis of Known Bacitracin Ophthalmic Ointment for Bacitracin by Two Different Methods

Trials	Assay by the Sodium Hypobromite–Phloroglucinol Reagent, mg/g	Assay by the Ninhydrin Reagent, mg/g
1	4.00	3.98
2	3.95	3.87
3	3.89	3.90
4	4.00	4.01
5	4.00	3.88
6	3.92	4.09
7	3.80	3.91
8	4.01	4.14
9	3.96	3.93
10	3.90	3.86
	$\bar{X}$ = 3.94	3.96
	SD = ±0.07	0.10

Color Formation—Pipet 4.0 ml of an aqueous solution containing 60–200 μg bacitracin/ml into a 20-ml glass test tube, and add 1.0 ml of sodium hypobromite solution. Place the tube in a boiling water bath for 5 min and then add 3.0 ml of phloroglucinol reagent. Stir and develop the color by placing the tube in the bath again for a further 10 min. A pink stable color is developed. Cool by placing the tube in cold water (20°) for about 10 min.

Pipet 2.0 ml of 95% ethanol into the tube (occasionally a faint opalescence develops which disappears when ethanol is added) and shake well. Determine the absorbance, A_p, of the solution at 505 nm in a 2-cm cell against a reagent blank using a suitable spectrophotometer. Concomitantly carry out the reaction with the reference standard bacitracin in a 20-ml glass test tube, and follow the procedure described for color formation.

By designating the bacitracin standard weighed out in milligrams as C_s and the absorbance of the standard solution produced as A_s, the bacitracin present in the ointment can be calculated from the equation:

$$\text{bacitracin (mg/g)} = \frac{A_p \times C_s}{A_s \times \text{sample weight (g)}} \quad \text{(Eq. 1)}$$

Multiplying this equation by the factor I, where I indicates the microbiological potency of the standard bacitracin in units per milligram, one gets units of bacitracin per gram of ointment.

TLC—Ointments containing bacitracin and neomycin as active compounds were extracted as described. Bacitracin was then determined after TLC because neomycin interferes with the determination of bacitracin using this color reaction.

TLC was carried out on 20 × 20-cm glass plates coated with an approximately 300-μm layer of silica gel GF_{254}[4] activated at 110° for 45 min. The plates were treated with the developing solvent of *n*-butanol–acetic acid–water–pyridine (30:22:38:6, v/v) (12) over the whole 20-cm length and finally dried with a hair drier.

Using a microsyringe, apply 200 μl (approximately 500–800 μg of neomycin sulfate and bacitracin) of standard and test solutions as two thin uniform strips on the starting line of a prepared TLC plate. Take care not to damage the silica gel layer, which is easily flaked off the plate. Leave a 6-cm length in the middle of the plate for the blank. Develop the plate in the developing solvent for about 2.5 hr and then dry it, first by removing most of the solvent with the hair drier and then by placing the plate in a vacuum oven at 50° and 5–10 torr for 30 min. This procedure avoids decomposition of bacitracin.

Viewing the plate under a UV lamp (254 nm), mark out the standard and test zones of bacitracin situated on the upper half of the chromatogram, with R_f about 0.86, and scrape them separately off the plate using a spatula. Neomycin spots were visualized by spraying with ninhydrin reagent, R_f about 0.65. Place each zone in a 50-ml centrifuge tube fitted with a ground-glass joint, and place an equally sized area of adsorbent from the middle 6-cm section of the plate (representing the adsorbent background) into a third tube for preparing the blank.

[4] Merck.

Table II—Comparative Assay of Bacitracin in Ophthalmic Ointments Containing 4 mg/g Bacitracin

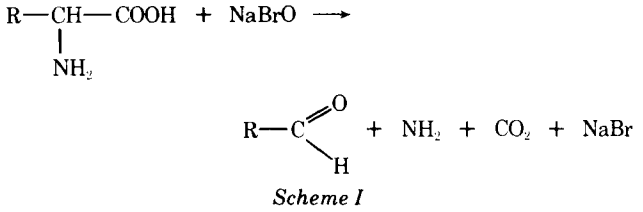
Sample	Colorimetric Method, %	Microbiological Method, %
1	102.3	113.6
2	99.1	107.2
3	97.3	104.1
4	96.8	97.8
5	100.4	96.6
6	98.2	98.8
7	97.7	92.7
8	99.4	103.2
	$\bar{X}$ = 98.9	101.75
	SD = ±1.81	±6.66
	RSD = ±1.81	±6.66

Into each tube, pipet 5.0 ml of pH 13 phosphate buffer (dissolve 100 mg of sodium phosphate dibasic dihydrate in 100 ml of 0.1 *N* sodium hydroxide, pH 12.7), and extract the active compound by placing the tubes on a mechanical shaker for 30 min. Centrifuge for 5 min at 3000 rpm.

Pipet 4.0 ml of each of the three supernatant solutions into three 20-ml test tubes, and carry out the color reaction in exactly the same manner as described under *Color Formation*, beginning with "... add 1.0 ml of sodium hypobromite solution." Bacitracin can be calculated from Eq. 1.

RESULTS AND DISCUSSION

By considering bacitracin as a compound containing aminocarboxylic acids of the type R—C(—NH_2)—H—COOH, the possible oxidation reaction is shown in Scheme I (14).

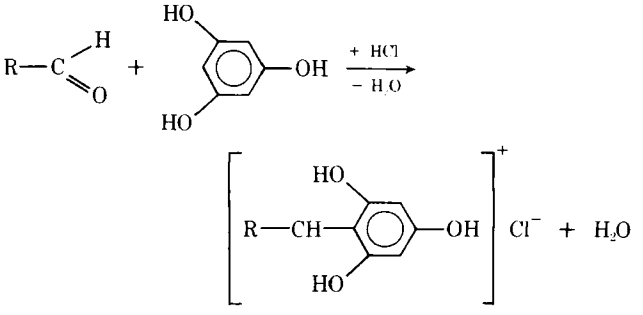


Scheme I

The α-aminocarboxylic acids are both deaminated and decarboxylated on treatment with alkali hypobromite. An aldehyde with one carbon atom less than in the original amino acid results.

The resulting aldehyde condenses with phloroglucinol in concentrated hydrochloric acid to yield pink-colored monomethine chloride (Scheme II) (15). The concentration optimum of the sodium hypobromite reagent was determined using concentrations of 1, 1.5, 2, 2.5, and 3% of active bromine. The best results were obtained with the reagent at 2%. The exact concentration of the reagent is very important, because large variations may lead to considerable changes in color intensity. Therefore, the concentration may be checked by prior iodometric titration of the bromine.

The reaction time was determined following the color development at the temperature of the boiling water bath. The results (Fig. 1) indicated that a heating time interval of only 5 min with the alkaline sodium hypobromite reagent is required to obtain



Scheme II

Table III—Assay of Bacitracin after TLC

Sample	Recovery, %
1	104.0
2	100.0
3	99.4
4	100.6
5	105.2
6	96.1

complete reaction. The optimum reaction time for the phloroglucinol reagent was 10 min, and only the 1% concentration in 31% hydrochloric acid was used.

The reaction reaches a maximum after 5 min and remains almost stable for several hours. This asset is very important for a method to be used regularly in quality control and for series determinations. Twenty-four hours after the beginning of the reaction, the maximum of the visible absorption spectrum remains constant at 505 nm while the absorbance shows a small increase of about 4% (Fig. 2).

Linearity—Concentration was found to be proportional to absorbance for the chromophore produced by reaction of bacitracin with the chromogenic reagent. A typical Beer's law plot is shown in Fig. 3. The linearity of the reaction was investigated in a concentration range of 15–100 μg/ml of bacitracin at the maximum at 505 nm. For concentrations lower than 10 μg/ml, the relationship is not linear.

Ten replicates of an eye ointment preparation, containing 4 mg (250 units) of bacitracin/g, gave an average of 3.94 mg/g with a relative standard deviation of 0.07 mg. The results were compared with those obtained by the ninhydrin method according to Langner and Heckel (16) (Table I). Data comparing the colorimetric method with the microbiological method on eight different samples of premix are presented in Table II.

The microbiological determination was carried out by the petri dish method (17, 18) using Difco Penassay seed agar (pH 6.6 after sterilization) as the culture medium and *Micrococcus flavus* (ATCC 10240) as the test microorganism.

The analytical method described here was used for the analysis of bacitracin in two commercial preparations after the TLC technique. The assay was performed on six samples, and the results obtained (Table III) show the suitability of this analytical procedure. The ninhydrin reagent gave unsatisfactory results after TLC.

Interferences—The method is relatively nonspecific and, therefore, is not stability indicating. In addition, any compound susceptible to oxidation by sodium hypobromite in an alkaline medium (such as other aminocarboxylic acid-containing compounds as well as neomycin) would probably react with phloroglucinol and, consequently, interfere with the determination of bacitracin using this procedure; suitable separation steps such as TLC or ion-exchange chromatography would be required. Figure 4 shows that the biological activity of bacitracin decreases more rapidly than is indicated by the colorimetric method.

A solution containing 1 mg/ml bacitracin in pH 13 phosphate buffer (see under *TLC*) was heated rapidly at 50° (water bath). Then an aliquot of this solution was neutralized, cooled, and diluted suitably for the colorimetric and microbiological methods. This solution was used as the standard for the further aliquots pipetted out in 5-min intervals, which were also neutralized, cooled, and diluted suitably. The colorimetric results were compared to those of the microbiological method.

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ACKNOWLEDGMENTS AND ADDRESSES

Received September 24, 1973, from the *Analytical and Development Department (Dispersa), Dr. E. Baeschlin Ltd., Winterthur, Switzerland.*

Accepted for publication August 9, 1974.

The author thanks Mrs. V. Cée and Miss E. Wyss for technical assistance.