Colorimetric Determination of Peptide Antibiotics: In-Process Assay of Cyclic Octapeptidic Antibiotics in Fermentation Broths

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Abstract □ A spectrophotometric method is presented for monitoring the biosynthesis of a new complex of cyclic octapeptidic antibiotics in fermentation broths. The method is based on the extraction of antibiotic from alkaline broth with butanol. An ion-pair, formed between the octapeptides and bromthymol blue, is extracted into chloroform from a solution buffered to pH 7.5. The absorbance of the colored solution is measured at 420 nm. Results are in good agreement with those obtained by microbiological assay. The method is also applicable to other peptidic antibiotics such as polymyxin B and gramicidin.

Keyphrases □ Octapeptide (cyclic) antibiotic biosynthesis—colorimetric monitoring in fermentation broths, ion-pair formation with bromthymol blue □ Antibiotic biosynthesis, cyclic octapeptide—colorimetric monitoring in fermentation broths, ion-pair formation with bromthymol blue □ Colorimetry—monitoring, in-process cyclic octapeptide biosynthesis, ion-pair formation with bromthymol blue

A new complex of cyclic octapeptidic antibiotics[1] (I) is produced by a strain of *Bacillus circulans*. The antibiotic has broad spectrum antibacterial activity and displays considerable antifungal and antiprotozoal activities (1–3). Proper control of the fermentation of I requires an analytical method that can monitor biosynthesis at any stage and that requires a comparatively short time.

With a microbiological assay[2], one can determine the amount of I in a fermentation broth about 48 hr after sampling. Chemical methods for the determination of polypeptidic antibiotics (4–8) are tedious and time consuming. In these laboratories, a rapid and simple colorimetric method was developed for estimating the amount of I in a fermentation broth. The reaction of bromthymol blue with I to form a chloroform-soluble colored complex is the basis of the method; the amount of colored complex is proportional to the amount of I present.

EXPERIMENTAL

Reagent grade *n*-butanol, chloroform, sodium chloride, sodium hydroxide, and absolute ethanol were used.

Phosphate buffer, pH 7.5, was prepared by dissolving 10 g of dibasic sodium phosphate, 2 g of monobasic potassium phosphate, and 5 ml of 85% phosphoric acid in 900 ml of distilled water. The pH was adjusted to 7.5 with 5 *N* sodium hydroxide, and the solution was diluted to 1 liter with distilled water.

Bromthymol blue, 0.15%, was prepared by dissolving 0.15 g of bromthymol blue and 0.15 g of anhydrous sodium carbonate in distilled water to give 100 ml of reagent.

The I standard, of known purity, was dissolved in distilled water and diluted to a concentration of about 500 μg/ml.

[1] EM49, Squibb Institute for Medical Research, New Brunswick, NJ 08903

[2] T. B. Platt, Squibb Institute for Medical Research, personal communication.

Table I—Effect of pH on Extraction of I—Bromthymol Blue Ion-Pair

pH	Absorbance at 420 nm
2.5	0.000
6.0	0.490
6.5	0.690
7.0	0.780
7.5	0.824
8.0	0.525
9.0	0.419
10.0	0.000
12.0	0.000

PROCEDURE

Extraction—A 5.0-ml sample of fermentation broth and 5.0 ml of standard I solution are pipetted into screw-type test tubes containing 2 g of sodium chloride, 5.0 ml of *n*-butanol, and 2 ml of 5 *N* sodium hydroxide solution. Each tube is covered with a plastic cap and shaken mechanically for 5 min. After centrifugation, 0.2 ml of the butanolic upper layer is pipetted into a screw-type test tube and the solvent is evaporated completely under a stream of air. Immersion of the tube in a water bath at about 70° is used to speed the evaporation of butanol.

Color Development—To each tube, 10.0 ml of pH 7.5 phosphate buffer is added. A reagent blank is prepared by pipetting 10.0 ml of pH 7.5 phosphate buffer into a screw-type test tube. To each tube, 1.0 ml of 0.15% bromthymol blue is added; then the contents are mixed. After the addition of 10.0 ml of chloroform, the tubes are covered with plastic caps and shaken mechanically for 5 min. The tubes are centrifuged at 2500 rpm for 1 min, and the aqueous upper layer is aspirated completely.

A 5.0-ml portion of the chloroform layer is transferred to a test tube containing 0.5 ml of absolute ethanol, and the contents are mixed. The absorbance of the sample and of the standard solutions is measured at 420 nm in a spectrophotometer[3] equipped with 1-cm cells, with the reagent blank in the reference cell. The amount of I is calculated from the concentration and the absorbance of the I standard.

RESULTS AND DISCUSSION

Positively and negatively charged ions can associate as uncharged ion-pairs. These neutral combinations exhibit considerably diminished water solubility and can be readily extracted into organic solvents. The principles of ion-pair formation were reviewed in detail previously (9).

Bromcresol purple, bromphenol blue, bromthymol blue, and methyl orange were tested for possible ion-pair formation with I. Of these indicators, only methyl orange and bromthymol blue formed a precipitate with I. At pH 7.5, both bromthymol blue and methyl orange couple with the positively charged basic molecule of I, but only the yellow I–bromthymol blue ion-pair can be extracted from aqueous solutions into chloroform and other organic solvents such as methylene chloride, ethylene dichloride, and ether. Carbon tetrachloride extracts only 8% of the color, and toluene extracts 25%. Cyclohexane, hexane, and heptane precipitate the ion-pair at the interface of the two phases. Butyl alcohol, isobutyl acetate, and

[3] Beckman DU.

Table II—Recovery of I from Broths

Added I, mg/ml of Broth	Recovery, %
2.0	101.0
1.6	101.0
1.2	99.0
0.8	99.4
0.4	100.0
0.2	101.0
0.1	98.5
0.0	0.0

ethyl acetate extract free bromthymol blue, thus producing very high blanks.

Study of the effect of pH of the aqueous solution on the reaction and extraction of the I–bromthymol blue ion-pair gave the results shown in Table I. Less color was extracted from acidic and alkaline solutions than from a neutral solution. An excessive amount of free bromthymol blue was extracted with chloroform from acidic solutions with pH up to 6.5. For example, the absorbance of the reagent blank at pH 6.0 was 0.730, contrasted with the absorbance of 0.100 from a pH 7.5 buffer. The amount of phosphate used in the preparation of the pH 7.5 buffer proved important. An increase in the phosphate content of the buffer solution led to an increase in the extraction of the ion-pair, but too high a concentration of phosphate in the buffer produced high reagent blanks.

The volume of pH 7.5 buffer used also affects the extraction of the ion-pair with chloroform. The best results were obtained with 10.0 ml of pH 7.5 buffer. For reproducible results, the phosphate buffer should be transferred with a volumetric pipet. The volume of chloroform proved to be unimportant; when the I–bromthymol blue ion-pair was extracted from 10.0 ml of phosphate buffer with 5, 10, 15, or 25 ml of chloroform, the same amount of color was extracted in each case.

Chloroform extracts of the ion-pair are cloudy. They can be clarified by centrifugation, but they become cloudy again on standing. The addition of anhydrous sodium sulfate clarifies the chloroform extract, but the readings of absorbance vary. Reproducible measurements of absorbance are obtained when chloroform extracts are clarified with absolute ethanol.

The I–bromthymol blue ion-pair shows maximum absorbance at 420 nm. Solutions containing up to 200 μg I/ml of chloroform, with an absorbance of 1.16, obey Beer's law.

Various solvents were tried for the extraction of I from aqueous solutions. Isobutyl acetate, ethyl acetate, benzene, isopentyl acetate, chloroform, and ethylene dichloride did not extract I. When the ratio of solvent to the aqueous solution was 1:1, methyl ethyl ketone extracted 30%, isoamyl alcohol extracted 19%, and isobutyl alcohol extracted 53% of the I present. Of the solvents tried, only *n*-butanol extracted about 96% of I. Complete extraction of I with *n*-butanol was obtained when an inorganic salt such as sodium chloride, ammonium chloride, or sodium sulfate was used to salt out the antibiotic. The use of one of these salts also prevented emulsification of the broth during extraction.

The effect of pH on the extraction of I was studied. The pH of solutions was adjusted with 5 *N* sodium hydroxide or 6 *N* hydrochloric acid to points between 1 and 12. Complete extraction of I occurred from solutions adjusted to pH 5 or higher; but at pH 1.0, only 57% of the I was extracted. Extraction of I from highly alkaline broths eliminates blanks from samples.

Table III—Reproducibility of Colorimetric Assay of I in Fermentation Broth

Sample	I, μg/ml of Broth	
	Run 1	Run 2
1	705	718
2	791	784
3	780	795
4	898	876
5	887	887
6	876	889

Table IV—Assay of I in Fermentation Broth by Colorimetric and Microbiological Methods

Duration of Fermentation, hr	Concentration of I, μg/ml of Broth			
	Chemical		Microbiological	
	Run 1	Run 2	Run 1	Run 2
0	0	0	<35	<35
6	56	54	<35	<35
14	89	90	<35	<35
22	488	496	545	474
30	570	560	636	682
38	600	583	682	750
46	660	680	727	818
54	689	710	727	750
62	793	800	727	818
70	902	894	795	886
78	896	900	750	886
86	893	880	886	796
96	901	892	909	886

The ratio of the volume of broth to the volume of butanol did not appreciably affect the efficiency of extraction. Compound I was extracted completely even when the ratio of broth to *n*-butanol was 2:1.

Recovery of I was studied by the addition of the antibiotic to a broth medium. As shown in Table II, complete recovery of I from the medium was obtained in the range tested.

Samples of fermentation broth were analyzed in duplicate. From the data given in Table III, it appears that results can be reproduced within ±2%.

Results from the colorimetric assay were compared with those from the microbiological assay of I. Broth samples were extracted in duplicate from solutions at about pH 12, and portions of the extracts were assayed both colorimetrically and microbiologically. *Bordetella bronchiseptica* (ATCC 4617) was used in the bioassay. As shown in Table IV, the results of the colorimetric assay were more reproducible than those of the microbiological procedure. When mean values for two assays by each method were compared, better correlation of methods was obtained at completion of the fermentation. The greater concentration of I shown by the microbiological assay could be attributable to fermentation products that do not form ion-pairs with bromthymol blue but are used up later in the process.

Broth samples were stable for at least 1 week when refrigerated at 5°. The I standard solutions showed a decrease of about 15% in color intensity after being kept at room temperature for 2 days. The butanolic extracts were stable for at least 1 week at room temperature.

This colorimetric assay of I in fermentation broths can be used to monitor the biosynthesis of other peptide antibiotics such as polymyxin B, gramicidin, and tyrocidine. Gramicidin, a component of the antibiotic tyrothricin, has to be separated from tyrocidine as described (10) before ion-pairs are formed. The $E^{1\%}_{1cm}$ of each polypeptidic antibiotic seems to depend on the number of free amino groups in the compound. The $E^{1\%}_{1cm}$ values of I, polymyxin B, gramicidin[4], and tyrocidine[5] are 772, 775, 193, and 319, respectively.

This new colorimetric assay can also be used for the determination of polymyxin B and gramicidin in pharmaceutical formulations.

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Colorimetric Determination of Isoniazid with 9-Chloroacridine

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Abstract □ A colorimetric method based on the interaction between isoniazid and 9-chloroacridine was developed. Analytical solutions are shaken for 30 min at 50°, and the absorbance is measured at 500 nm. The procedure is sensitive for isoniazid in the 10^{-5} *M* range. The method was applied to the analysis of isoniazid in pharmaceutical dosage forms and found to be comparable to the USP XVIII assay. Preliminary investigations suggested that the procedure is useful for the determination of free isoniazid in urine and plasma samples containing isoniazid metabolites.

Keyphrases □ Isoniazid—colorimetric analysis with 9-chloroacridine □ 9-Chloroacridine—colorimetric reagent for determination of isoniazid □ Colorimetry—analysis, isoniazid with 9-chloroacridine

The interaction of isoniazid (isonicotinic acid hydrazide) with 9-chloroacridine to give highly colored solutions has been observed in this laboratory. This observation led to the development of a new colorimetric method for determining isoniazid with 9-chloroacridine. The reagent was shown previously to be applicable to the colorimetric analysis of primary aromatic amines and aromatic hydroxylamines (1–4).

Existing analytical procedures for the assay of isoniazid include visible and UV spectrophotometry, polarography, and titrimetry. The colorimetric methods involve the interaction of the drug with 1-chloro- or 1-fluoro-2,4-dinitrobenzene (5, 6) and/or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (7). Although highly sensitive and specific, these procedures have the disadvantages of instability of color formation and preparation of multireagent solutions. Vanillin has also been used as a chromogenic reagent in the analysis of isoniazid (8).

A procedure involving the hydrolysis of isoniazid to hydrazine, with subsequent color development with *p*-dimethylaminobenzaldehyde, was reported (6). This method is subject to interference by aldehydes and/or ketones. UV spectrophotometry (6) and polarography (6) also have been utilized in the analysis of microgram quantities of isoniazid. The USP XVIII method (9) involves an iodometric titration and is laborious and time consuming.

This paper presents a new colorimetric method for determining microgram quantities of isoniazid with 9-chloroacridine. The method has been successfully applied to the analysis of the drug in pharmaceutical dosage forms. In addition, preliminary investigations suggest that the procedure is useful for the determination of free isoniazid in urine and plasma samples.

Table I—Analysis of Known Isoniazid Mixtures for Isoniazid

Mixture	Components[b]	Isoniazid[a] Found, $M \times 10^{-5}$	Isoniazid[a] % of Theory
1	Isoniazid Pyridoxine hydrochloride	4.00 ± 0.04[c]	100.00
2	Isoniazid Chlorobutanol	3.96 ± 0.04	99.00
3	Isoniazid Aminosalicylic acid	3.95 ± 0.06	98.75
4	Isoniazid Acetylisoniazid Isonicotinic acid Isonicotinuric acid	3.98 ± 0.02	99.50
5	Isoniazid 1,2-Diacetylhydrazine	3.98 ± 0.02	99.50
6	Isoniazid *N*-Acetylhydrazine	3.95 ± 0.03	98.75

[a] Based upon three replicate determinations of each solution. [b] Final concentration of isoniazid and all other components in the mixture, except aminosalicylic acid, was 4×10^{-5} *M*; aminosalicylic acid was present in a final concentration of 2×10^{-5} *M*. [c] Confidence limits at $p = 0.05$.

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers[1,2]. Matched cells with a 1-cm optical path were used.

Reagents and Chemicals—9-Chloroacridine[3] was used as the chromogenic reagent. It was recrystallized before use by the method of Albert (10). Isoniazid[4] was purified by recrystallization from 80% methanol and dried under reduced pressure, mp 172–173°. Isonicotinic acid[5] was used as received. Acetylisoniazid, α-ketoglutaric acid isonicotinoylhydrazone, pyruvic acid isonicotinoylhydra-

[1] Perkin-Elmer model 450.
[2] Bausch and Lomb Spectronic 20.
[3] Eastman Organic Chemicals, Rochester, N.Y.
[4] Matheson, Coleman and Bell, East Rutherford, N.J.
[5] Aldrich Chemical Co., Milwaukee, Wis.