5. All silica-type glidants were found to produce a decrease in mean tablet weight along with a tendency toward an increase in coefficient of variation for the same size die fill when used with the spray-dried lactose/microcrystalline cellulose blend. This phenomenon was attributed in large measure to the extremely high bulk volumes of the silica-type additives which have the effect of increasing the bulk volume of the entire powder blend, thereby decreasing effective tablet weights.

6. The statistical testing of coefficients of variation revealed no difference between the Stokes model B-2 tablet machine and the Colton model 216 tablet machine at the 95% confidence level.

7. Glidants *A* and *B* proved effective in concentrations as low as 0.1% by weight when added to plain microcrystalline cellulose. No increase in glidant activity was observed with concentrations beyond 0.5% by weight.

8. For the same die fill, tablet weights decreased and coefficients of variation tended to increase as tablet machine speeds increased when glidants *A* or *B* were added to microcrystalline cellulose. As would be expected, a powder fluidity becomes more critical when press speed is increased.

9. Although calcium acetate has proved to be an effective glidant in the cement industry, it did not increase tablet weights when added to microcrystalline cellulose or the spray-dried lactose/microcrystalline cellulose blend. It did appear to decrease coefficients of variation, but no explanation is offered.

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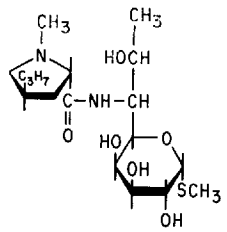
Automated Assay for the Antibiotic Lincomycin

By GEORGE C. PRESCOTT

A chemical assay for the antibiotic lincomycin has been developed which is based on the colorimetric determination of methanethiol generated from the acid hydrolysis of the methylthioglycosido group of the antibiotic. Fermentation beers and production samples as well as purified materials can be assayed by this procedure. Automation of this method increased the number of (completed) assays per man day from about 30 to about 150. Application of the assay to a series of standard solutions has given a mean recovery of 101 per cent and a standard deviation of 5.1 per cent. Analysis of a centrifuged beer containing added increments of lincomycin has given a mean recovery of 95.5 per cent and a standard deviation of 6 per cent.

LINCOMYCIN[1] is a medium spectrum antibiotic having the structure shown in I (1).

In 1962 the author developed an assay method which was based on the colorimetric determination of methanethiol generated from the acid hydrolysis of the methylthioglycosido group of the antibiotic (2). A particular advantage of this assay was that the thiol was separated from the interfering background material by distillation before it was reacted with the color reagent. Fermentation beers and production samples as well as purified products were assayed by this procedure. Early attempts to automate this assay with an automatic analyzer[2] were un-



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The author acknowledges the help and suggestions of Mr. Andres Ferrari of the Technicon Corp., Chauncey, N. Y., particularly in using polyethylene and glass tubing for methanethiol gas and for the gas separator hook-up; the technical assistance of C. J. Kleinhuizen, Jr., and his positioning of the mixing coils and fittings and the use of positive air pressure; and is indebted to J. R. Marsh for the drawing of the automatic analyzer flow diagram.

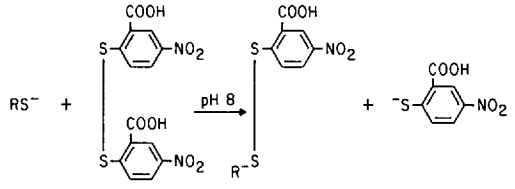
[1] Marketed as Lincocin by The Upjohn Co., Kalamazoo, Mich.

[2] AutoAnalyzer, Technicon Controls, Inc., Chauncey, N. Y.

successful due to absorption of methanethiol gas by the Tygon tubing. This difficulty was eliminated by allowing the gas to contact only glass or polyethylene tubing. Other important improvements included the use of a water-soluble color reagent, better positioning of the mixing coils and gas separator, and the use of positive pressure on the system. Automation increased the number of (completed) assays per man day from about 30 to about 150.

The reactions involved in the assay are: acid cleavage of lincomycin to yield methanethiol, separation of the thiol, and reaction with a disulfide color reagent at pH 8.0 to release the highly colored anion. Full color development is almost instantaneous at room temperature.

The reaction of thiols with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is shown in Scheme I.



Scheme I

EXPERIMENTAL

Instrumentation.—Technicon sampler No. 2, proportioning pump, colorimeter, and recorder.

Reagents.—*A.*—Sulfuric acid, approximately 12 *N*.

B.—Color reagent, 0.01% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Dissolve 100 mg. of the reagent in 5 ml. of ethanol and dilute to 1 L. with water. The reagent appears to be stable for at least 1 month. In fact it is desirable to allow the reagent to stand for 24 hr. or longer as the slight haze which develops when the water is added disappears during this time.

C.—Tris buffer 0.1 *M*, pH 8.0. Dissolve 12.12 Gm. of 2-amino-2-hydroxymethyl-1,3-propanediol in 54.5 ml. of 1.0 *N* hydrochloric acid and dilute to 1 L. with water.

D.—Polysorbate 20.[3]

Procedure.—Set up the equipment as shown in the flow diagram (Fig. 1). Use a timing cam with the sampler 2 which allows 30 sec. for the sample and 90 sec. for wash or a rate of 30 samples/hr. Standardize the automatic analyzer as shown in the test manual.

The following points in technique have been found to be essential for a satisfactory assay.

(*a*) Tygon tubing is unsatisfactory for handling methanethiol gas. Glass and polyethylene tubing have been found to be satisfactory and *must* be used where indicated in the diagram.

(*b*) The gas separator must be connected in the manner indicated in the diagram which allows the aliquot for analysis to flow by gravity. The normal procedure of pumping this aliquot is unsatisfactory due to the Tygon manifold tubing.

(*c*) The mixing coils must be placed in a vertical position rather than in the usual horizontal position. The fittings must also be positioned in the approximate angle indicated.

(*d*) A positive air pressure of 3 lb./sq. in. greatly improves the bubble pattern and eliminates any back pressure in the system.

(*e*) As with most assays with this automatic analyzer the bubble pattern is greatly improved by using a small amount of surface-active agent in the reagents. The following amounts of polysorbate 20 seem to be about optimum under present conditions: 0.05 ml./L. in the acid, 0.1 ml./L. in the buffer, and 0.05 ml./L. in the water wash. The polysorbate 20 should be added to the acid and buffer on the same day that they are used.

Sample Preparation.—Prepare samples to contain from 25–150 mcg./ml. of lincomycin base per ml. of water. The pH of the solution is not critical since the sample will be hydrolyzed with strong acid. Highly colored samples cause no difficulty since the methanethiol gas is separated from the liquid before the colorimetry. However, solutions containing large amounts of carbonates or other materials which evolve gas on acidification must be degassed by acidifying at room temperature (lincomycin is not hydrolyzed at room temperature). Samples containing more than traces of volatile solvents should be evaporated and redissolved in water. Since the sample pick-up tubing is only 0.034 in. i.d., it is obvious that samples containing suspended solids must be clarified.

Standard Curves.—*Standard Curve for Purified Samples.*—Prepare standards to cover the assay range using lincomycin hydrochloride monohydrate primary standard. It has been found convenient to prepare standards containing 30, 60, 90, 120, and 150 mcg./ml. of lincomycin base equivalent in water. The concentration of lincomycin in each standard solution is calculated as lincomycin base, using a conversion factor based on the lincomycin base equivalent contained in the lincomycin hydrochloride monohydrate reference standard. These standards appear to be stable for several weeks at room temperature.

Standard Curve for Fermentation Beer Samples.—Prepare standards to cover the assay range in the same manner as described above, except use spent beer for the diluent in place of water. This spent beer must show no activity by either bioassay or chemical assay. It should be diluted with water to about the same dilution as the unknown samples.

Calculations.—Plot a transmission curve from the recorded peak averages of standards (Fig. 2). Draw a line connecting the transmission peaks of the standards and determine the concentration of each unknown from this curve. In most cases the curve is linear over the assay range, and it is equally satisfactory to determine the slope of the standard curve and calculate the concentration of the unknowns using this value. However, when the standard curve is not linear or does not pass through the origin the unknowns must be read directly from the curve. During the course of a long run it is necessary to correct for instrumental drift. This is the chief reason for running a standard for every 3

[3] Marketed as Tween 20 by the Atlas Powder Co., Wilmington, Del.

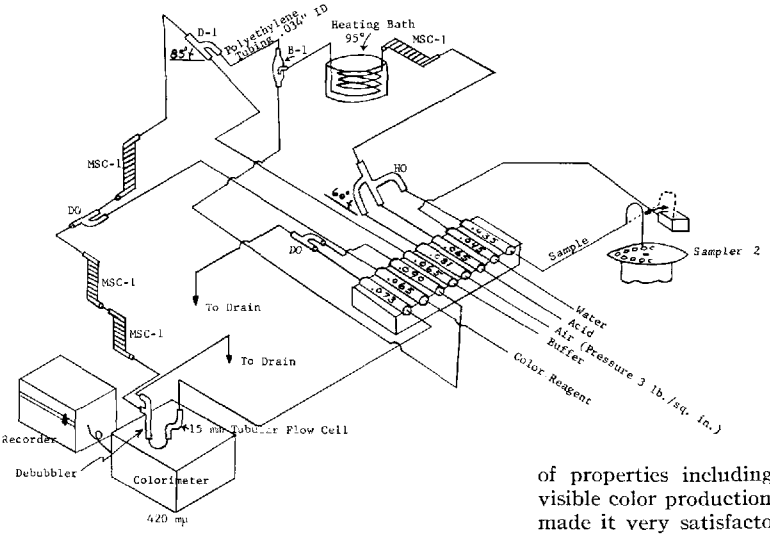


Fig. 1.—Automatic analyzer flow diagram for lincomycin using sampler 2. All glass fittings are designated by the Technicon AutoAnalyzer code.

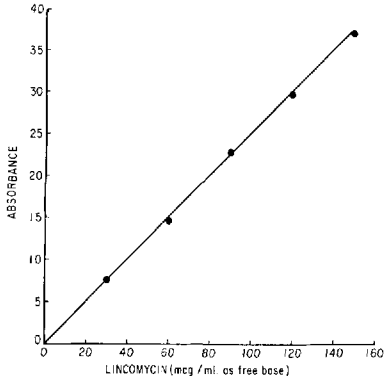


Fig. 2.—Standard curve for lincomycin assay.

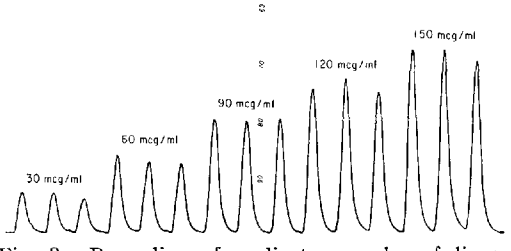


Fig. 3.—Recording of replicate samples of lincomycin standards.

samples throughout the entire run. This instrumental drift is inherent to the automatic analyzer and has been discussed by Thiers and Ogelsby (7).

RESULTS AND DISCUSSION

Choice of Reagent.—Several colorimetric reagents for thiol groups were investigated before the present reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (3), was chosen. This reagent possessed a combination of properties including high sensitivity, stability, visible color production, and water solubility which made it very satisfactory for this assay. Some of the reagents investigated and rejected are: *N*-ethylmaleimide (4), bis(*p*-nitrophenyl)disulfide (5), 2,2-diphenyl-1-picrylhydrazyl (6), and sodium nitroprusside.

Specificity.—As would be expected, lincomycin analogs and metabolic products having a hydrolyzable thiol group give full molecular response in the assay. This applies to methylthiolincosaminide (1) as well as other lincomycin-related antibiotics (8). Most of the ingredients of the fermentation media do not show any response. These include methionine, ethionine, corn steep liquor, and Wilson's peptone No. 159. Blackstrap molasses, however, exerts a slight positive bias when it is added to standard samples. This may be the reason for the positive bias of fermentation samples when they are calculated from standards prepared in water. This bias is small and somewhat variable, averaging around 5%. Better agreement between the 2 assays is obtained by calculating the beer samples from a standard curve in which the crystalline standard is diluted with spent beer as described under *Standard Curves*.

Precision.—The plot of concentration *versus* absorbance is virtually linear at least over the concentration range of 25–150 mcg./ml. of lincomycin. At the described rate of assay there is no sample interaction as shown in the recording of replicate samples (Fig. 3). Application of the method to a series of standard solutions has given a mean recovery of 101% and a coefficient of variation of 5.1%. Analyses of a centrifuged beer containing added increments of lincomycin have given a mean recovery of 95.9% and a coefficient of variation of 6.0%.

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