Colorimetric Assay of Nystatin

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A colorimetric method to assay nystatin both as powder and in pharmaceutical preparations is described. Nystatin is hydrolyzed with sodium hydroxide; the yellow color produced is extracted from a buffered solution into chloroform and measured colorimetrically. Comparison with microbiological assay is shown in a degradation experiment.

NYSTATIN is usually assayed by microbiological methods (1–4). A spectrophotometric method has been reported (5), but it has been known in our laboratories to be subject to ultraviolet absorbing interferences and is not suitable for stability studies.

A yellow color is produced by heating nystatin with sodium hydroxide and this may lead to a colorimetric assay method (6). Direct measurement of the color was tried in this laboratory, but the results did not agree too well with the microbiological assays. Unterman (7) reported a similar method recently.

It was thought that extraneous color was present in the solution after hydrolysis, and color produced by nystatin alone could be extracted into another solvent. Chloroform was found to be the best solvent. It was demonstrated that by measuring the color extracted into chloroform, the results were reproducible and agreed well with microbiological assays, and that degradation of nystatin could be followed by this method.

Preliminary experiments showed that the color produced by the basic hydrolysis of nystatin was unstable in both basic and acidic solution. However, the color was stable for at least 16 hours if the pH was kept at about 6.5. In order to maintain this pH, a citrate buffer was used and found to be satisfactory. Acid neutralization using bromthymol blue as indicator also was tried, but this was abandoned because the indicator was extracted into chloroform.

The time of color development was studied. The intensity of color reached a maximum in 2 minutes at boiling-water temperature and decreased rapidly upon longer heating (see Fig. 1); therefore it was necessary to quench the reaction in an ice bath after maximum color was obtained.

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One 10-ml. portion of chloroform extracted about 95% of the color produced by nystatin, and the color was virtually completely extracted with two 10-ml. chloroform extractions. The chloroform extract was turbid due to the presence of water, but the turbidity was removed by adding alcohol. Drying agents were tried but were not as satisfactory.

Different solvents for nystatin were tried, among them methanol and isopropyl alcohol. Although hydrolysis occurred in all the solvents tried, dimethylformamide was chosen because nystatin dissolved in it readily.

EXPERIMENTAL

Reagents

Ethanol, U.S.P. (or SD3A, if colorless); dimethylformamide, reagent grade; chloroform, reagent grade; 1 *N* sodium hydroxide.

Citrate buffer: prepared by dissolving 210.15 Gm. citric acid and 29.4 Gm. sodium citrate, both reagent grade, in water to make 1 L. of solution

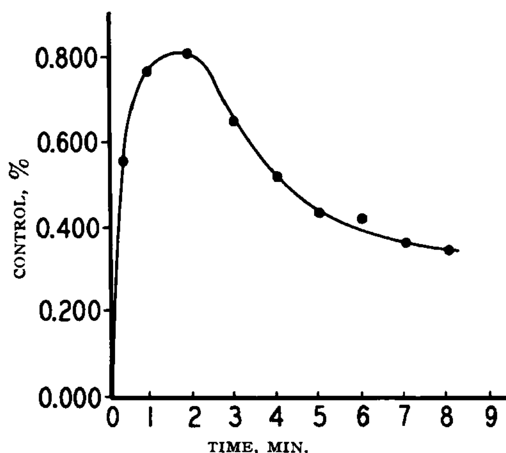

Fig. 1.—Color development of basic hydrolysis of nystatin (5080 units in 5 ml. aliquot).

The pH of this solution should be 2.3 ± 0.2. If not, 10 *N* sodium hydroxide or 60% citric acid was added to adjust the pH.

Dilute buffer: prepared by diluting 20 ml. of the citrate buffer to 100 ml. with water.

Procedure

A solution of nystatin in dimethylformamide, representing about 700 u./ml., was prepared as directed in *Preparation of Samples*. To a test tube was added 3 ml. 1 *N* sodium hydroxide. Then a 5-ml. aliquot of nystatin solution was added along the side of the test tube in such manner that the nystatin solution formed a layer floating on top of the sodium hydroxide solution. The solutions were quickly mixed by a slight shaking motion, and immediately the test tube was placed in a boiling water bath for exactly 2 minutes. Then the test tube was cooled in an ice bath for 2 minutes, and the contents immediately transferred to a 60-ml. separator containing 6 ml. of dilute buffer. The test tube was rinsed with water, combining the rinsings in the separator. The yellow color was extracted twice with 10 ml. of chloroform, shaking $^1/_2$ minute each time, and the chloroform extracts were withdrawn into a 50-ml. volumetric flask. Ten milliliters of ethanol was added to the flask, and chloroform was added to the mark. After mixing, the absorbance at 385 mμ was measured on a Beckman-DU spectrophotometer in 1-cm. cells, using chloroform to adjust to zero absorbance.

Duplicate aliquots of each sample solution were assayed simultaneously with duplicate aliquots of a standard, and the potency of the sample was calculated from that of the standard by a simple proportion. Although the procedure was standardized, the samples were always assayed with a standard in order to obtain best results.

Preparation of Samples

Powder.—A weighed amount of powder, representing about 70,000 u. was shaken with 50 ml. of dimethylformamide in a 100-ml. volumetric flask for 20 minutes, and dimethylformamide was added to volume.

Cream.—An amount containing about 70,000 u. of nystatin was weighed into a 50-ml. centrifuge tube and was shaken twice with 10 ml. of dimethylformamide for 1 minute. After each shaking the tube was centrifuged for 15 minutes, and the super-

TABLE I.—ASSAY OF NYSTATIN AS THE POWDER AND IN PHARMACEUTICAL PREPARATIONS

Sample	Colorimetric Assay[a]	Average	Microbiological Assay[b]	Spectrophotometric Assay[c]
Powder 1, u./mg.	3,510			
	3,550	3,530	3,580	3,630
Powder 2, u./mg.	3,890			
	3,840			
	3,860			
	3,840	3,860	3,830	3,870
Powder 3, u./mg.	3,750			
	3,770			
	3,720	3,750	3,330	3,780
Powder 4, u./mg.	3,610			
	3,720	3,670	3,500	3,860
Powder 5, u./mg.	3,350			
	3,260	3,310	3,220	...
Powder 6, u./mg.	3,810			
	3,750	3,780	3,690	...
Powder 7, u./mg.	3,760			
	3,740	3,750	3,550	...
Cream 1, u./Gm.	128,200			
	127,700	128,000	117,000	...
Cream 2, u./Gm.	123,600		128,000	...
Ointment 1, u./Gm.	101,600			
	100,600	101,100	108,000	...
Ointment 2, u./Gm.	110,300			
	113,100			
	111,300	111,600	116,000	...
Capsule 1, u./cap.	284,100		306,000	...

[a] Each value represents an individual assay with an independent sample preparation. [b] Assayed by the agar-diffusion method using *Saccharomyces cerevisiae*. [c] Assayed by measurements of absorbance at 291 mμ (8).

TABLE II.—DEGRADATION OF NYSTATIN AT 50° C.

Time, wks.	Colorimetric Assay (u./mg.) Control, 0°	Stability, 50°	Per cent of Control[b]	Microbiological Assay (u./mg.)[a] Control, 0°	Stability, 50°	Per cent of Control[c]
0	3504	...	...	3360	...	...
3	...	3044	86.7	3290	2820	85.7
5	...	2914	83.0	3300	2770	83.9
7	3504	2740	78.1	3170	2380	75.1
9	3524	2673	76.2	3020	2370	78.5
11	3533	2564	73.1	3270	2270	69.4
17	3480	2300	65.5	3030	2040	67.3
20	...	2384	67.9	2980	1940	65.1

[a] Assayed by the agar-diffusion method using *Saccharomyces cerevisiae*. [b] Calculated from the average of the assays of the control samples. [c] Calculated from the corresponding assay of the control sample.

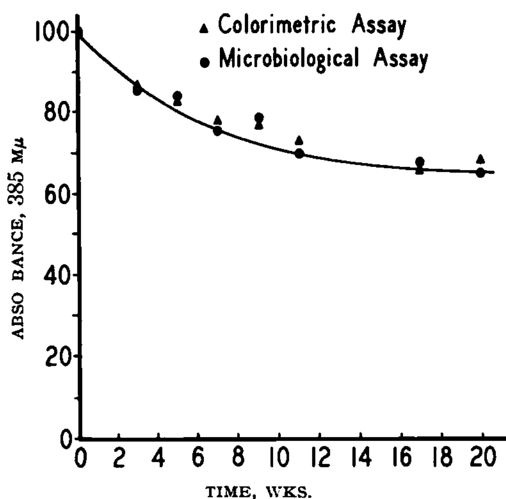

Fig. 2.—Degradation of nystatin at 50° C.

natant solution transferred into a 100-ml. volumetric flask. The volume was made up with dimethylformamide.

Ointment.—Same as *Cream*, but without centrifuging.

Capsule (containing tetracycline phosphate complex).—An amount of solids containing about 70,000 u. of nystatin from a mixture of the contents of five capsules was weighed into a 50-ml. centrifuge tube containing about 200 mg. of magnesium chloride hexahydrate. Twenty-five milliliters of water was added, and the tube shaken for 1 minute. After centrifuging, the water solution was discarded. The tube was washed with 25 ml. of water, centrifuged, and the water again discarded. The residue was then dissolved in small portions of dimethylformamide and transferred into a 100-ml. volumetric flask. The volume was made up with dimethylformamide.

All the nystatin solutions were prepared in low actinic glass volumetric flasks.

Degradation Study

One sample of powder (Powder 1) was chosen for a degradation study. Samples of 500 mg. each were placed in 2 × 9-cm. open vials spread out to a thickness of 0.5 cm. and stored in an oven at 50 ± 2°. The samples were withdrawn periodically over 20 weeks and assayed by both the colorimetric and microbiological methods.

RESULTS AND DISCUSSION

Results of assays of nystatin as the powder and in pharmaceutical preparations are shown in Table I along with comparisons to the microbiological assays and to the spectrophotometric assays (8) for four samples of powder. It can be seen that the agreement is good. The colorimetric assays show good reproducibility with a standard deviation of about 2%. The standard deviation for the microbiological assay is about 3.3% for the powders, and about 3.7% for the creams, ointments, and capsule.

Results of the degradation study are shown in Table II. A plot of the stability assays expressed as percentage of the control assay *vs.* time is shown in Fig. 2. Since the microbiological control assays varied quite widely due to differences in sensitivity of the organism, the percentages of the microbiological stability assays are calculated from the corresponding control assays. The percentages of the colorimetric stability assays, on the other hand, are calculated from the average of the control assays because deviation in the colorimetric control assays was small, the largest difference being only 1.5%. From the plot in Fig. 2, it can be seen that all the points lie closely on the same curve, demonstrating the colorimetric assay to be a stability assay.

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