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Evaluation of Nystatin Stability Using Tristimulus Colorimetry

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Abstract □ A tristimulus reflectance spectrophotometer was used to examine the color changes of nystatin during accelerated stability studies, and a relationship was observed between the loss of microbiological potency and the change in color during thermal degradation. By substitution of the measured tristimulus values in the Kubelka–Munk equation, the remission function was calculated and resulted in a linear response with time. Application of the technique to bulk raw materials and formulated products is demonstrated, and uses of the technique are discussed.

Keyphrases □ Colorimetry—nystatin, thermal degradation, relationship between loss of microbiological potency and color change □ Degradation, thermal—nystatin, colorimetry, relationship between loss of microbiological potency, and color change □ Nystatin—thermal degradation, colorimetry, relationship between loss of microbiological potency and color change □ Antifungal agents—nystatin, thermal degradation colorimetry, relationship between loss of microbiological potency and color change

The most frequent use of surface color measurements in the pharmaceutical industry has been the monitoring of color stability of coated tablets (1). Alternative uses of the procedure have been described, particularly in preformulation and stability studies. These applications generally involve the determination of the discoloration exhibited by a particular drug–excipient mixture or formulation. By this means, the incompatibility of various mixtures was reported (2–6), and the stability of active ingredients in formulated products was determined (7). Prediction of the shelflife of tablets also may be assessed by this technique (8, 9). A comprehensive review of the pharmaceutical applications of tristimulus colorimetry and diffuse reflectance spectroscopy was published recently (10). The stability of the polyene antibiotic nystatin was evaluated in these laboratories by monitoring the color change at elevated temperatures. The color change at a solid surface is measured quantitatively by diffuse reflectance spectrometry, and monochromatic light generally is used to illuminate the sample. Alternatively, illumination may be by a white light source, and the values of each of the three primary color components (the tristimulus values) of the reflected light are determined. This technique usually is referred to as tristimulus colorimetry.

For nystatin, a visual relationship between the degree of browning observed and the loss of microbiological potency was demonstrated previously[1]. Therefore, tristimulus colorimetry was used to quantitate the observed color changes since the measurement of the tristimulus values closely parallels the color discrimination obtained with the human eye.

THEORY

To quantitate the observed degradation of nystatin, it was necessary to derive a suitable equation so that a relationship between the color and intensity of the reflected light, as described by the tristimulus values, and the concentration of colored material in the reflecting surface was defined. Kubelka and Munk (11, 12) derived an equation to describe the phenomenon of diffuse reflectance. For the special case of an infinitely thick opaque layer, it is:

$$f(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty} = \frac{k}{s} \qquad \text{(Eq. 1)}$$

where R_∞ is the diffuse reflectance of the layer relative to a nonabsorbing standard, k is the molar absorption coefficient, s is the scattering coefficient, and $f(R_\infty)$ is the remission function. In practice, this requirement will be satisfied by a layer of material several millimeters deep.

The color of light reflected from the sample surface is described by the measured tristimulus values X, Y, and Z. The Y value corresponds to the Munsell value, which measures the degree of sample darkening, without regard to hue or chroma. The tristimulus values are used to calculate the chromaticity coordinates x, y, and z according to $x = X/(X + Y + Z)$, $y = Y/(X + Y + Z)$, and $z = Z/(X + Y + Z)$.

Tristimulus values are measured relative to a nonabsorbing standard, usually a white ceramic tile. For this study, the color of a sample before and after degradation was compared. This comparison may be achieved if the color of the degraded material is determined using the material prior to degradation as the standard. However, this procedure introduces practical difficulties and necessitates a large supply of original material. To overcome these difficulties, all materials were measured against a white tile as the standard, and the relative color change was calculated from the expression x_0/x_t, where x_0 is the initial chromaticity coordinate of the sample and x_t is the chromaticity coordinate at time t.

Substitution of these values into the Kubelka–Munk equation gives:

$$f(R_\infty) = \frac{(1 - x_0/x_t)^2}{2x_0/x_t} \qquad \text{(Eq. 2)}$$

[1] U. Nager, unpublished data.

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Table I—Reproducibility of Nystatin Preparations

Sample	Cream			Powder		
	X	*Y*	*Z*	*X*	*Y*	*Z*
1	64.2	67.5	56.3	82.4	86.8	74.1
2	64.4	67.5	56.5	82.4	86.8	73.8
3	64.2	67.4	56.2	81.8	86.4	73.6
4	64.3	67.5	56.7	82.3	86.7	74.0
5	64.2	67.3	56.3	81.9	86.6	73.8
6	64.3	67.5	56.9			
7	64.1	67.5	56.8			
8	64.4	68.0	56.1			
9	63.9	67.5	56.4			
10	64.2	67.5	56.4			
Mean	64.2	67.5	56.5	82.2	86.6	73.8
SD	0.14	0.18	0.26	0.28	0.18	0.19
CV	0.21	0.26	0.46	0.34	0.20	0.25
95% Confidence limit	64.1–64.3	67.4–67.6	56.3–56.7	81.9–82.5	86.4–86.8	73.6–74.1

Similar equations may be written involving the *y* and *z* chromaticity coordinates.

EXPERIMENTAL

Tristimulus Value Measurements—*Powders*—To determine the tristimulus values of degraded nystatin powder, it was necessary to devise a sample holder. A nickel crucible cover[2] proved to be suitable. The recess in the cover was filled loosely with powder and leveled off with a spatula. The powder then was compressed into the recess by pressing a similar cover on top, resulting in a powder bed that was ~2 mm thick. The underside of this second cover was highly polished so that a smooth powder surface remained after compression.

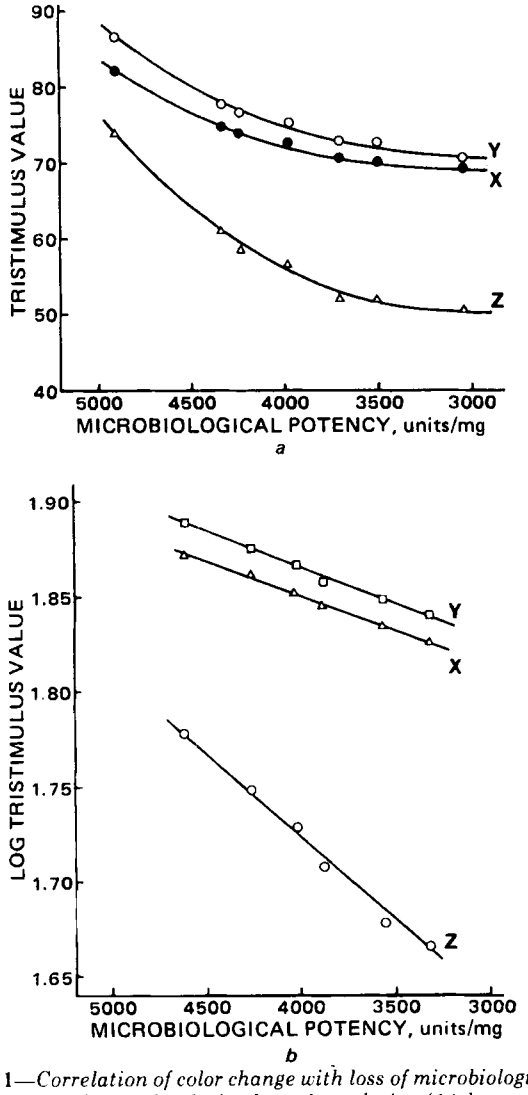

Figure 1—*Correlation of color change with loss of microbiological potency for nystatin powder during heat degradation (14 days at 75°).*

[2] Catalog item 3328-M10, A. H. Thomas, Philadelphia, Pa.

Ointments and Creams—For these formulations, a suitable sample holder consisted of a 3-mm thick polytef spacer, with a hole 30 mm in diameter, sandwiched between two clear glass microscope slides. To prepare a sample for color measurement, one microscope slide was placed beneath the spacer, and the 30-mm orifice was overfilled slightly with the ointment or cream to be tested. The second microscope slide then was placed on top, and the sandwich was gently squeezed together. By this means, all air bubbles were eliminated from the sample surface.

Measurement of the tristimulus values was accomplished by clipping either sample holder over the sample port of the colorimeter[3]. All measurements were made against a standard white tile[4] as the reference and with the nonspecular insert fitted.

Correlation of Potency with Color Change—Nystatin powder was heated at 75 and 85° in open dishes and in sealed screw-top bottles. Stored material was sampled at 24-hr intervals for 14 days. The color of the samples was determined as already described. The microbiological potencies of the same samples were determined by the agar-plate procedure using *Saccharomyces cereviseae*[5] (SQ 1600) and referenced against the current nystatin reference standard.

Degradation of Nystatin Powder and Formulations—Ointment and cream formulations were filled into Araldite-lined aluminum tubes and stored in thermostatically controlled environments at 20, 32, and 40°. The color of samples was assessed after 3, 6, and 12 months of storage. Additional examination of the samples at 40° was undertaken after 1 and 2 months of storage.

Powders were stored in humidity ovens[6] under ambient and 75% relative humidity (RH); 75% RH was achieved with a saturated aqueous solution of sodium chloride in contact with excess solid phase. Degradation was studied at 75, 85, and 100° by withdrawal of samples at 24-hr intervals for color determination.

RESULTS AND DISCUSSION

Before proceeding with the experimental work, the reproducibility of the proposed methods for tristimulus value determination was appraised. Tristimulus value measurements on representative fractions from the same batch of ointment or raw material (Table I) demonstrated good reproducibility for both sample types. A direct comparison of the tristimulus values with microbiological potency (Fig. 1*a*) demonstrated that an unprecedented quantitative relationship existed between the two parameters during the heat degradation of nystatin. The results represent the observed degradation over 14 days at 75°. Similar curves were obtained at higher temperatures. Furthermore, a linear correlation was obtained when the tristimulus values were plotted as logarithmic functions (Fig. 1*b*). Therefore, quantitation of the color change of the antibiotic is an alternative method of assessing the microbiological stability of nystatin.

Further examination of the heat-degraded nystatin by reversed-phase

[3] Color-Eye (Signature model), Instrument Development Laboratory.
[4] Reference Standard Daylight C, Instrument Development Laboratory.
[5] Squibb culture collection.
[6] Laboratory Thermal Equipment, Oldham, United Kingdom.

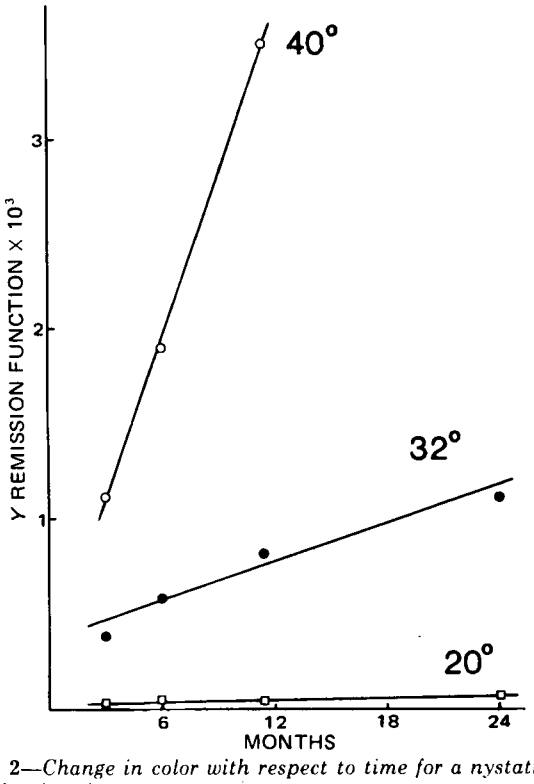

Figure 2—*Change in color with respect to time for a nystatin–steroid–lidocaine ointment.*

high-performance liquid chromatography[7] indicated that the observed color change also was related to the chemical decomposition of nystatin. A chemical decomposition of 13% was observed during the 14-day degradation period.

Ointments and Creams—To determine the applicability of the method to ointment and cream formulations, nystatin–steroid preparations were examined at intervals throughout the normal stability program, and the Kubelka–Munk functions were calculated from the tristimulus values obtained. A plot of the Kubelka–Munk remission function *versus* time (Fig. 2) for a nystatin–steroid–lidocaine ointment demonstrates a linear response over the time period considered. Either the X or Y function may be used with essentially similar results. The slope of the remission function *versus* time graph represents the degradation rate constant k. The k values obtained for the degradation of the ointment at 20, 32, and 40° were evaluated in accordance with the Arrhenius equation. A plot of log k *versus* $1/T$ resulted in a linear graph, thus suggesting that accelerated stability evaluations at 40° are valid for the prediction of room temperature stability (Fig. 3).

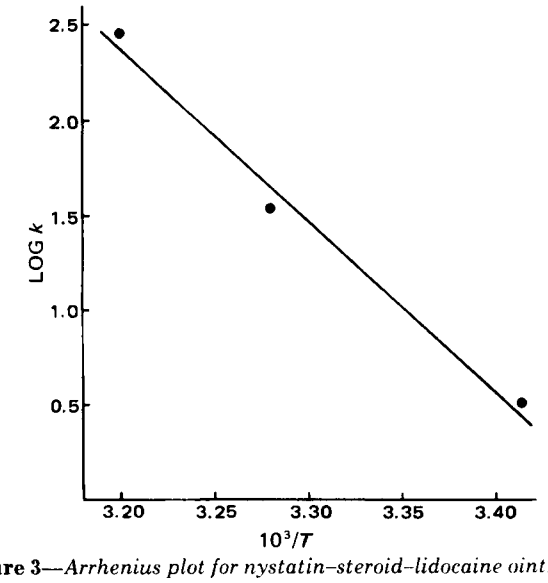

Figure 3—*Arrhenius plot for nystatin–steroid–lidocaine ointment.*

[7] P. R. Wood and J. R. Salmon, presented at the 35th International Congress of Pharmaceutical Sciences (F.I.P.), Dublin, Ireland, 1975.

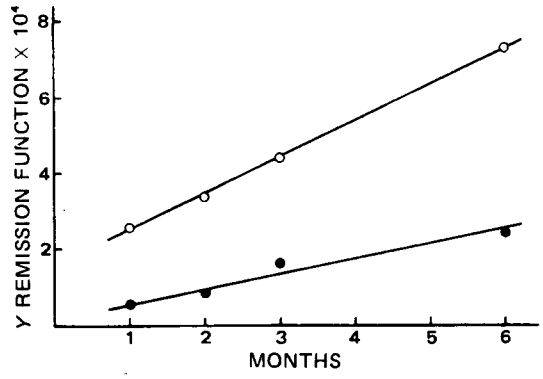

Figure 4—*Comparison of the color change of two nystatin creams. Key:* ●, *with added antioxidant; and* ○, *without antioxidant.*

Discoloration of the antibiotic in two cream formulations is shown in Fig. 4, and a linear response with time again is apparent. The difference in the slope values indicates the difference in stability of the two formulations (one contained an antioxidant) and confirmed data previously obtained in these laboratories using microbiological assays.

Accelerated stability testing of creams and ointments at temperatures higher than 40° has not been attempted.

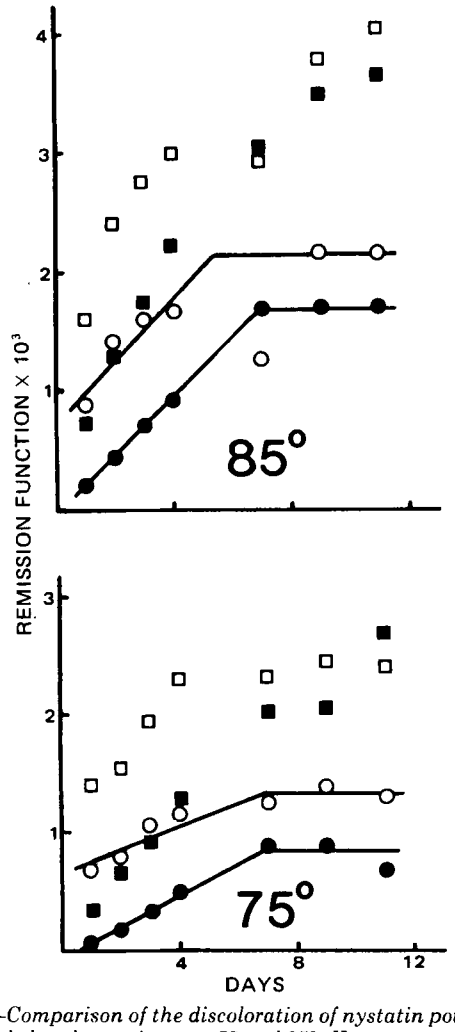

Figure 5—*Comparison of the discoloration of nystatin powder stored in open and closed containers at 75 and 85°. Key:* □, *open dish,* X *remission function;* ■, *closed container,* X *remission function;* ○, *open dish,* Y *remission function; and* ●, *closed container,* Y *remission function.*

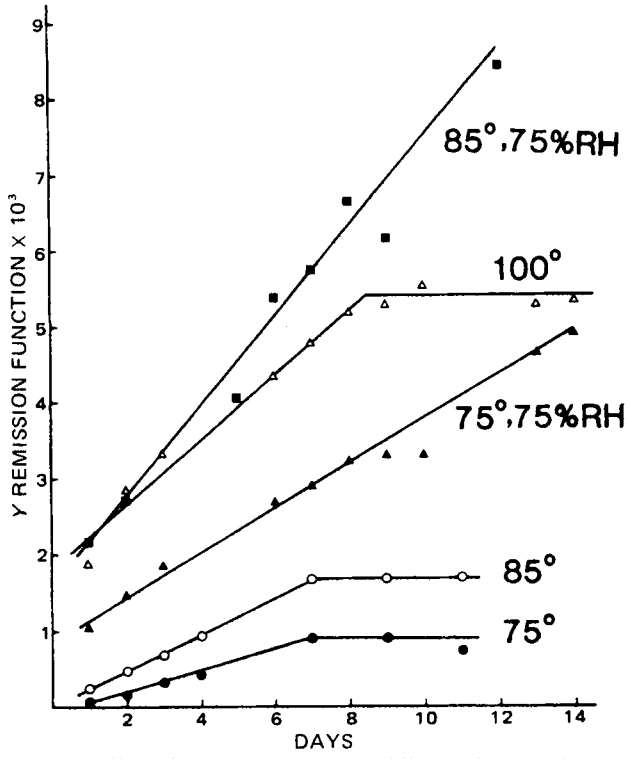

Figure 6—*Effect of temperature and humidity on the rate of nystatin powder discoloration.*

Bulk Raw Material—With the ointment and cream formulations, which are stored in sealed containers during stability monitoring, only one variable (temperature) is possible. However, with solids, the material may be stored in open or closed systems; with open containers, humidity may be included as another variable parameter. Bashkovich *et al.* (13) reported an increase in the rate of nystatin inactivation in a humid atmosphere.

To assess the effect of these variables on the discoloration of nystatin, the antibiotic was stored in open dishes and in sealed screw-top vials at

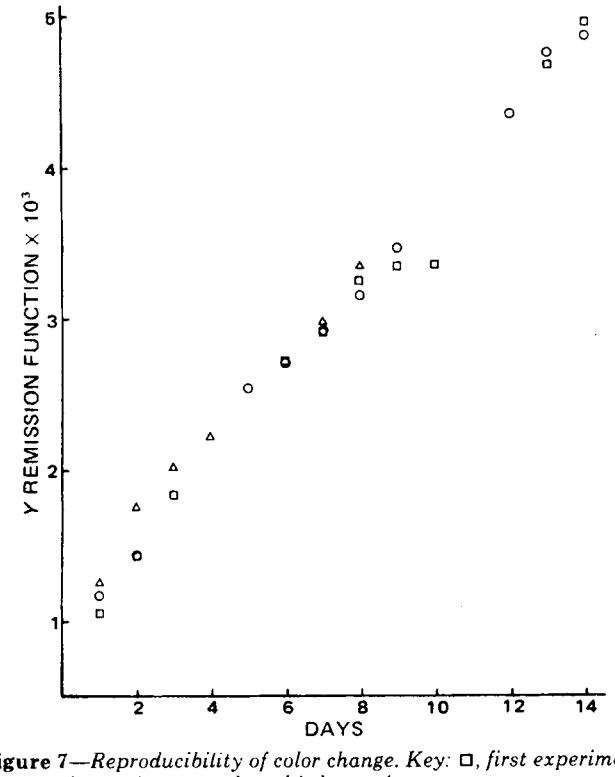

Figure 7—*Reproducibility of color change. Key:* □, *first experiment;* ○, *second experiment; and* ●, *third experiment.*

Table II—Reproducibility of Technique

Assay	Slope × 10^4	Correlation Coefficient
1	4.57	0.9935
2	4.79	0.9964
3	5.23	0.9928

a series of temperatures. In additional experiments, the powder was stored in an atmosphere of 75% RH in open dishes at elevated temperatures. The color change was determined daily, and the Kubelka–Munk remission functions were calculated from the results.

The use of open and closed containers is compared in Fig. 5, which shows the change in remission function with time. The plots for each type of container demonstrate that essentially similar results were obtained regardless of the temperature. Comparison of the tristimulus values of the degraded nystatin samples, determined before and after thorough mixing of the material, showed little difference, indicating that the color change was not a surface phenomenon but instead took place throughout the entire material.

The effect of moisture on nystatin degradation is demonstrated in Fig. 6. Not only was the degradation rate increased, but the plateau effect, noted with samples stored at ambient relative humidity, was eliminated under humid conditions. The nature of the plateau effect is not understood fully, but it is thought to be due in part to a loss of moisture from the nystatin samples, all of which contained about 5% moisture prior to degradation. This moisture level is normal in the manufactured product.

Only samples stored under humid conditions resulted in a linear change of the Kubelka–Munk remission function throughout the 14-day period. The most satisfactory correlation coefficient was obtained for the nystatin degraded at 75° and 75% RH. To assess the reproducibility of the degradation rate under these conditions, one batch of nystatin was degraded on three separate occasions during 3 months. The results (Fig. 7) show little scatter and indicate the robustness of the procedure. Calculation of the respective slopes and correlation coefficients for each set of points (Table II) demonstrated the reproducibility of the technique.

The linear response thus allows calculation of an actual degradation rate, which is advantageous when comparing factors affecting nystatin stability, as in preformulation studies, or when comparing the stability of bulk nystatin from different sources. Previous investigations were accomplished microbiologically (13–15). However, the decrease in potency observed during the degradation period was nonlinear and allowed only qualitative comparisons. With the alternative procedure described in this paper, it is possible to compare materials and formulations quantitatively, an objective previously unattainable.

If the application of this novel technique to other antibiotics proves successful, the tristimulus colorimetric technique should be established firmly in pharmaceutical analysis.

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