Rapid Spectrophotometric Method for the Assay of Nystatin in Feeds

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A rapid method for the assay of nystatin in feeds at levels as low as 5 ppm has been developed. Samples were extracted with methanol, and nystatin was directly quantified in crude extracts on the basis of the characteristic trough that appeared at 308 nm when the normal absorption spectrum in the range 260−360 nm was submitted to third-derivative processing. Precision data suggested an overall standard deviation of 5.6%. The accuracy of the assay was found to be 96.7 ± 2.4%, whereas the linearity was quite acceptable in the range examined. Because of its rapidity and increased sensitivity and specificity, the new method might be a good alternative to the official AOAC microbiological method.

Keywords: *Nystatin; fungicide; spectrophotometric determination*

INTRODUCTION

Nystatin is a polyene antifungal antibiotic that is of particular interest to the feed industry because it exhibits remarkable action against a wide range of pathogenic and nonpathogenic yeasts and fungi. It has been widely used as a feed additive at levels of 50−100 ppm to control and treat crop mycosis and mycotic diarrhea of chickens and turkeys caused by *Candida albicans* (*Feed Additive Compendium*, 1983). As it is poorly absorbed, the antifungal effect of nystatin is mainly exhibited within the gastrointestinal tract (Huber, 1977).

Nystatin possesses a carboxyl group that is present as a zwitterion, a primary amino group, and a large number of free hydroxyl groups. The presence of the hydroxyl groups and the zwitterion is responsible for the comparative insolubility of the compound in organic solvents. It also exhibits a characteristic ultraviolet absorption spectrum with three main peaks at around 291, 304, and 318 nm due to the presence of four consecutive conjugated double bonds in its 38-atom unconjugated lactone ring (Oroshnik et al., 1955). The net absorbance of the central peak, which may be determined by subtracting the average absorbance of the minima on each side of the peak itself, has formed the basis for the spectrophotometric assay of the antibiotic in pharmaceutical preparations (Thomas, 1976). However, direct spectrophotometric measurement of nystatin at its absorption maxima is unsuitable for stability studies because of interference by absorbing degradation products. A correction for nonspecific absorption in the near-ultraviolet region is required (Amer et al., 1975), and for this reason the method cannot be applied for the analysis of more complicated samples such as the medicated feeds.

The assay of nystatin in medicated feeds is carried out by microbiological methods using *Candida tropicalis* (Haney et al., 1963), *C. albicans* (Platt et al., 1969), or *Saccharomyces cerevisiae* (AOAC, 1965) as the test organism. A common feature in these methods is the need to prepare a blank feed extract for each particular sample to compensate for the biological activity exhibited by other coextracted compounds. Early microbiological methods included very complex sample preparation procedures to compensate for this effect (Haney et al., 1963; AOAC, 1965, 1970). Modifications to earlier methods (Platt et al., 1969; Platt and Itkin, 1974) have led to the development of the present official AOAC method (AOAC, 1990), which is considerably simplified. The sample is directly extracted with methanol, whereas the blank feed extract is prepared by autoclaving a portion of the extract at 121 °C, a temperature at which inactivation of nystatin occurs after brief heating. The method represents a major improvement over earlier methods, but it may still be considered time-consuming and labor-intensive. It requires overnight incubations, and serious analytical errors may be introduced without separate standard curves for each type of feed at each concentration (Platt et al., 1969). It is further relatively insensitive as concentrations of nystatin lower than 50 ppm cannot be determined.

In this paper a simple, rapid, and more sensitive approach to the determination of nystatin in medicated feeds is presented. Extraction of nystatin is carried out with methanol, whereas discrimination of the analyte in the crude feed extract is directly performed through the use of derivative spectrophotometry. Quantification is carried out on the basis of the intensity of the third-derivative trough at 308 nm where interferences from other coextracted compounds are negligible.

MATERIALS AND METHODS

Instrumentation. A Shimadzu Model UV-160A double-beam spectrophotometer with 1-cm absorption cells was used for nystatin measurements; a Retsch KG (Haan, Germany) laboratory mill equipped with a 0.5-mm screen, a vortex blender (Heidolph, Germany), and a Centra-MP4 IEC centrifuge (Needham Heights, MA) were used for sample treatment.

Normal spectra were obtained between 260 and 360 nm at a scanning speed of 1500 nm/min. Third-derivative spectra were produced by digital differentiation of the normal spectra, according to convolution method, with 17−25 data points

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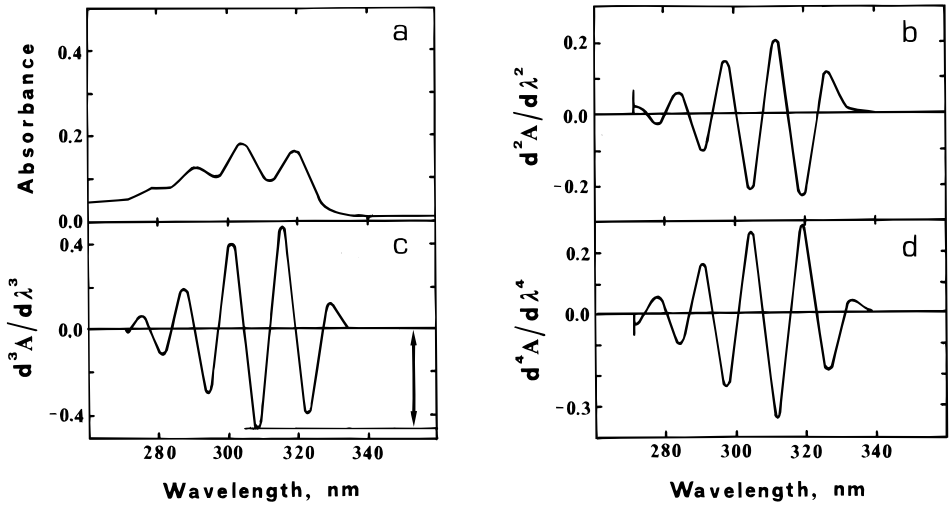

Figure 1. Normal (a) and corresponding second- (b), third- (c), and fourth-order (d) derivative spectra of 2 μg of standard nystatin/mL of methanol.

around each wavelength. The derivative wavelength difference ($\Delta\lambda$), which depends on the setting of the key entry N and the measuring wavelength range, was 6.3 nm for an $N = 9$ setting and a λ range $\leq$100 nm.

Reagents. Analytical grade dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile (ACN), and methanol (MeOH) were obtained from Merck (Darmstadt, Germany); nystatin was purchased from Sigma Chemical Co. (St. Louis, MO).

Stock solutions of nystatin were prepared by weighing ca. 25 mg of the compound and dissolving in and diluting to 25 mL with dimethylformamide. Aliquots of these stock solutions were further diluted with methanol to give working solutions in the range 0.04−4.0 μg of nystatin/mL. All solutions were protected from light and prepared fresh daily.

Feed Samples. Various commercial formulations for laying hens and broilers, obtained from the local market, were employed.

Analytical Procedure. A 1-g quantity of ground sample was transferred to a 50-mL screw-capped centrifuge tube and accurately weighed. A 20-mL volume of methanol was added, and the tube was vigorously vortexed for 2 min and centrifuged for 30 s at 1000*g*. A 1-mL aliquot of the clear supernate was transferred into a 15-mL screw-capped tube to which a 3-mL volume of methanol was also added. The tube was briefly vortexed, and its content was first submitted to normal spectrophotometry in the range 260−360 nm against water and then to third-derivative processing using a derivative wavelength difference of 6.3 nm.

Determination. The prepared series of working solutions was also submitted to normal spectrophotometry and third-derivative processing. A calibration curve was constructed by plotting values of trough depth at 308 nm, as they were printed on the instrumental chart in arbitrary units, versus known concentrations of nystatin. The concentration of nystatin in sample extracts was calculated using dilution factor and slope and intercept data of the computed least-squares fit of the calibration curve.

RESULTS AND DISCUSSION

The effect of second-, third-, and fourth-order derivative processing on a normal UV spectrum of 2 μg nystatin/mL of methanol is shown in Figure 1. Derivative processing enhances sharp features, transforming the normal spectrum into a series of peaks and troughs that may be used to resolve and locate the analytical band. In the second-order derivative spectrum, the trough appearing at 304 nm and the peaks at 297 and 312 nm correspond, respectively, to the maximum and the inflection points of the conventionally recorded central analytical band at 304 nm. In the fourth-order derivative function, the above-specified trough is becoming a peak and the peaks troughs, whereas in the third-order derivative spectrum the peak appearing at 300 nm and the trough at 308 nm correspond to the inflection points of the analytical band, the absorption maximum being now represented by the zero-crossing at 304 nm. Considering the significantly higher response of the third-order derivative function, the trough at 308 nm appeared to be an optimum reference point for nystatin determination. Regression analysis of the data obtained by running a series of working solutions showed the depth of this third-derivative trough to be linear in the range examined (Table 1).

Table 1. Raw Data and Regression Equation of Calibration Curve for Nystatin Quantification by Third-Derivative Spectrophotometry

concn of standard, μg/mL	mean ($n = 6$)[a] trough depth ± SD	RSD, %
0.04	0.008 ± 0.0009	12.3
0.08	0.019 ± 0.0006	3.0
0.16	0.037 ± 0.0008	2.2
0.50	0.109 ± 0.0008	0.7
0.80	0.181 ± 0.0006	0.3
1.00	0.226 ± 0.0008	0.4
2.00	0.456 ± 0.0012	0.3
4.00	0.903 ± 0.0038	0.4

[a] Depth of trough is expressed in arbitrary units; regression equation: $Y = (-2.28 \times 10^{-4}) + (0.226)X$, where Y represents the depth of the trough and X the concentration of standard nystatin solution in μg/mL; correlation coefficient 0.9999.

Figure 2 shows that while the normal spectrum of a feed sample containing 100 ppm of nystatin gives no analytical information due to strong overlap of the analytical band by interfering bands of other absorbing compounds, third-order spectra can lead to qualitative and quantitative results even when samples contain as low as 5 ppm of nystatin. Because of the improved resolution and band discrimination, third-order absorbance differentiation permits, thus, direct quantification of nystatin in the methanolic feed extracts. Further cleanup of these crude extracts was not required since the derivative transformation of the conventional analytical band at 304 nm virtually eliminated spectral interferences arising from other compounds.

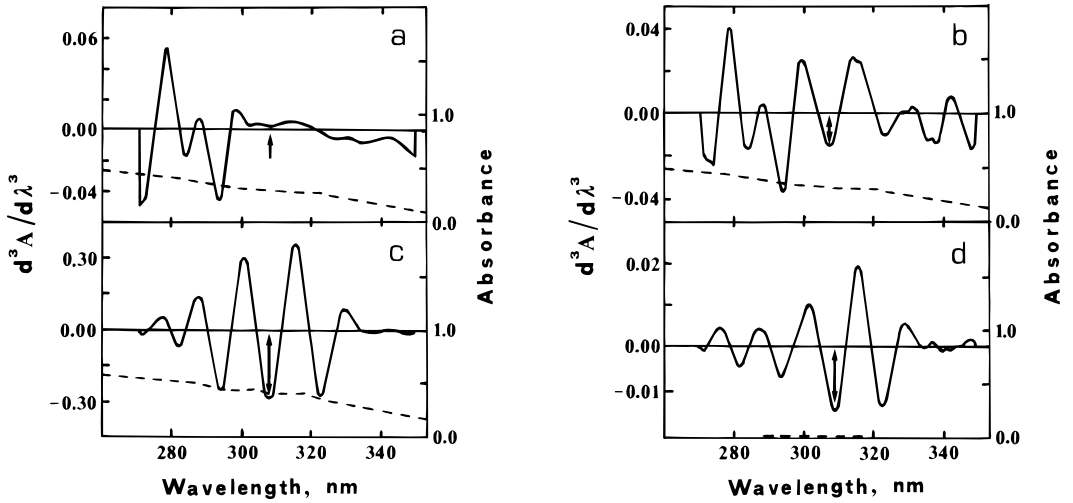

Figure 2. Normal (---, right *Y*-axis) and corresponding third-derivative (—, left *Y*-axis) spectra of a blank feed sample (a), feed samples containing 5 (b) and 100 ppm (c) of nystatin, and a sample of a 5 μg/mL standard solution (d). All samples were subjected to the same analytical procedure.

Table 2. Stability with Time of Solutions of Standard Nystatin in Selected Solvents (1.67 μg/mL)

	depth of trough at 308 nm (arbitrary units)				
solvent	0 min	30 min	60 min	90 min	150 min
H_3PO_4, 1 M	0.215	0.191	0.179	0.164	0.140
NaOH, 1 M	0.260	0.249	0.246	0.232	0.219
H_2O	0.239	0.229	0.224	0.214	0.175
ACN	0.225	0.171	0.160	0.151	0.149
MeOH	0.378	0.376	0.374	0.371	0.366
DMF	0.307	0.307	0.305	0.301	0.293
DMSO	0.271	0.266	0.259	0.253	0.237
ACN/H_2O, (1/1 v/v)	0.348	0.341	0.339	0.332	0.322
MeOH/H_2O, (1/1 v/v)	0.324	0.322	0.318	0.311	0.300
ACN/ H_3PO_4, 1 M (1/1 v/v)	0.317	0.293	0.280	0.270	0.255

To increase the sensitivity of the measurement, the instrumental parameter $\Delta\lambda$, which highly affects the intensity of the derivative fluctuations, was investigated. Generally, increasing $\Delta\lambda$, which is the derivative wavelength increment over which the derivative is obtained, decreases the noise level and increases the sensitivity of the measurement; however, if the value of $\Delta\lambda$ is too large, the spectral resolution deteriorates (Fletouris et al., 1993; Botsoglou et al., 1993). Therefore, the optimum value of $\Delta\lambda$ should be determined by taking into account the noise level, the resolution of the spectrum, and the sensitivity of the measurement. Various values of $\Delta\lambda$ were tested, and the value of 6.3 nm was selected as optimum for the assay of nystatin in feeds.

Although the third-derivative function could normalize any constant background absorption or gradual background variation produced by coextracted compounds, efforts were also directed toward finding alternative extraction solvents that might offer better selectivity and sensitivity toward nystatin than methanol. Investigation of the extraction efficiency of various selected solvents showed the adequacy of dimethylformamide, dimethyl sulfoxide, and methanol for the extraction of feeds, corroborated the better selectivity of methanol, and demonstrated the loss in extraction efficiency that occurred when relatively high proportions of water were added in the above solvents. It also showed that although acetonitrile could not extract nystatin at all, aqueous acetonitrile could extract it quantitatively. Experiments on nystatin stability further pointed out (Table 2) that methanol is the solvent in which nystatin exhibits its highest stability and maximum derivative response. In some of the solvents examined, such as water and acetonitrile, nystatin exhibited decreased stability (Table 2), but these results should be treated with caution because subsequent dilution of the aqueous or acetonitrile solutions with acetonitrile or water, respectively, led to derivative responses higher than those accounted for by the dilution. This effect should be due to the limited solubility of nystatin in those media in which the compound exists as a micellar suspension. It has been stated that the ultraviolet absorption spectra of micellar suspensions, in general, exhibit a peculiar type of spectral "degradation" similar to the effect of severe steric hindrance, which has caused more than one investigator some perplexity (Thomas, 1976).

The effect of time of methanol extraction on the recovery of nystatin from the commercial feed formulations employed was also determined. Incomplete recovery was noted when the extraction time was limited to 1 min. However, extractions of 2 min or longer led to quantitative results.

The accuracy of the method was studied by analyzing six replicates of 1-g samples of a feed formulation that had been spiked with standard nystatin at six fortification levels ranging from 5 to 150 ppm. The results are summarized in Table 3. To further estimate the overall recovery of the method, the concentrations of the nystatin added (X) and those found (Y) were submitted to least-squares and regression analysis. Analysis data showed that the relationship between X and Y could be described by a linear regression ($Y = -1.24 + 0.967X$, $r = 9993$) and, therefore, the slope (0.967 ± 0.024) of this regression line might be used as an estimate of the overall recovery ($96.7 \pm 2.4\%$) of the method. The limit of quantification, defined as the lowest nystatin concentration for which recovery and precision data were deemed acceptable, was fixed at 5 ppm. This limit is far lower than that attained by previous methods.

The precision of the method was also studied by assaying, on each of three different days, six replicates of 1-g feed samples spiked with nystatin at a 30 ppm level. To estimate the components of variance, the concentrations found were subjected to "analysis of variance and expected mean squares for the one-way classification-balanced design" (Wernimont, 1987).

Table 3. Accuracy Data Based on Analysis of Feed Samples Spiked with Nystatin at Different Levels

spiking level, ppm	no. of samples	nystatin found, ppm	mean value ± SD, ppm	RSD, %	recovery, %
5.0	6	4.6, 5.0, 5.3, 3.2, 4.5, 4.1	4.5 ± 0.7	15.2	89.0
10.0	6	8.2, 8.7, 7.9, 8.7, 9.2, 9.3	8.7 ± 0.5	5.7	86.7
30.0	6	27.1, 27.1, 27.5, 27.1, 26.6, 28.0	27.2 ± 0.4	1.6	90.8
50.0	6	45.1, 45.6, 47.1, 48.0, 46.1, 43.1	45.8 ± 1.6	3.4	91.7
100.0	6	96.9, 96.9, 95.6, 95.2, 94.7, 96.5	96.0 ± 0.9	0.9	96.0
150.0	6	139.4, 147.3, 142.9, 149.9, 140.7, 142.9	143.9 ± 3.7	2.5	95.9

Table 4. Precision Data Based on Analysis, at Different Days, of a Series of Feed Samples Spiked with Nystatin at 30.0 ppm Level

day	no. of samples	nystatin found, ppm	mean value ± SD, ppm	RSD, %
1	6	27.1, 27.1, 27.5, 27.1, 26.6, 28.0	27.2 ± 0.4	1.6
2	6	26.6, 24.4, 24.4, 27.1, 24.9, 30.2	26.3 ± 2.0	7.8
3	6	28.8, 28.0, 25.3, 26.2, 25.8, 26.2	26.7 ± 1.2	4.7
total			26.7 ± 1.5	5.5

Variance Estimates

source	RSD, %
within-day	4.4
between-day	5.8
overall	5.6

Precision statistics showed that the within-day and between-day precision, expressed as percent relative standard deviation, were 4.4 and 5.8%, respectively (Table 4). They also suggested that the overall precision, which is in fact the overall uncertainty of a single determination, was 5.6%.

Possible interferences with nystatin analysis from the presence of each of several antibiotics or drugs that are, frequently, added in feeds and/or used for treatment of diseases, such as chlortetracycline, tetracycline, oxytetracycline, monensin, furazolidone, furaltadone, nitrofurazone, carbadox, chloramphenicol, amprolium, sulfadiazine, sulfamethazole, and tylosin, were also examined. It was found that only sulfadiazine and sulfamethazole interfered with the analysis; the presence of each of these drugs at a 125 ppm level resulted in a 10% underestimation when feeds contained 50 ppm of nystatin, due to some overlapping of the third-derivative spectra at 308 nm.

In conclusion, the results of the present study show that the use of third-derivative spectrophotometry provides an accurate, precise, and sensitive method for the determination of nystatin in feeds. There is no need for costly reagents, and sample preparation is minimal so that a single analyst can easily process 10 samples in 1 h. Specially trained staff are not required, and the equipment needed is easily accessible as most modern spectrophotometers allow instant generation of derivative spectra. These advantages render the method valuable for routine analysis.

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