DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

MEMORANDUM

Date: December 29, 1995

From: Division of Biometrics and Production Drugs
Biometrics Branch, HFV-124

Subject: Review of studies intended to establish Natamycin as a mold retardant of *Aspergillus parasiticus*, *Penicillium rubrum*, and *Fusarium moniliforme* for up to 14 days in broiler chicken feed.

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Through: Chief, Biometrics Branch, HFV-124 12/29/95 MAL

C-002234-A-0000 (F/L 06/30/95; CVM 06/30/95)
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SUBMISSION:

The company submits a petition pursuant to section 409(B)(1) of the Federal Food, Drug, and Cosmetic Act with respect to Natamycin as a mold retardant of *Aspergillus parasiticus*, *Penicillium rubrum*, and *Fusarium moniliforme* for up to 14 days in broiler chicken feed.

Utility studies including laboratory dose titration studies in each of the molds and field studies for the purpose of confirming the dose titration results are reviewed below. Also included in the utility package are studies validating the method of respirometer CO_2 production and O_2 consumption measures as indicators of mold growth. The validation studies are not a subject of this review in accordance with wishes of the primary reviewer.

I. DOSE TITRATION STUDIES:

A. Study Design:

Five studies were conducted. Four studies were conducted using chicken mash as the substrate. There was one for each of the three types of mold and a repeat

study using *A. parasiticus*. These four studies in mash had Natamycin concentrations as follows: 0, 5, 10, 15, and 20 grams per ton. Two-hundred gram samples of sterilized feed were prepared at each test concentration by contaminating them with the appropriate quantity of mold spores and then the appropriate concentration of Natamycin. Four replicate flasks were formed of 20 gram aliquots each. The experiments differed in pre-incubation time, i.e., time prior to the beginning of respirometer recordings. In the initial study using *A. parasiticus* the moisture content of the feed was 17% and the initial spore contamination varied widely from 5 to 8 x 10^3 spores per gram. The repeat study using *A. parasiticus* used a higher feed moisture content of 17.38% and the initial spore contamination was more tightly controlled to 5000 spores per gram as it was in the other mash studies. The intended moisture content in the other studies was 17%; the measured moisture content for *P. rubrum* was 17.2% and for *F. moniliforme* was 17.7%.

After the pre-incubation period, the flasks were hooked up to the respirometer and O_2 consumption and CO_2 production were measured over a span of at least fourteen days. The amount of gas was accumulated in four-hour blocks.

Chicken pellets were used as the substrate in the fifth study and the doses used were the same as those in the other studies minus the 15 gram per ton dose. The feed was contaminated naturally as well as by introduced *A. parasiticus*. Mold growth on the pellets was evaluated subjectively rather than by means of respirometer. A colored picture of one representative petri dish from each dose showing mold growth on the feed is provided.

B. Statistical Review:

The accumulating totals of O_2 consumption and CO_2 production were analyzed at time points approximately a day apart, i.e. after four or five four-hour intervals, in the studies of mold growth on mash. The analysis consisted of a one-way analysis of variance with treatment being the one fixed effect in the model. A least significant difference test (LSD) at the alpha level 0.05 was performed to indicate differences among the treatments if the overall F tests were significant. Although LSD results are presented for both O_2 and CO_2, only the analysis of variance tables for the oxygen could be found in the submission. We verified that the analysis of variance tests and the LSD results the sponsor provided are similar to those obtained by using SAS. Graphs of the accumulating gas consumption or production average for the four replicate flasks by treatment group are provided. They show

fairly clear dose differences beginning at about 200 hours. Since there is a high degree of correlation among accumulating values and these studies covered only the growth and not the decay phase, the relevant variable for choosing a dose is the terminal value which represents total mold growth. In some cases, however, the final determinations have too much variation to allow differences to be detected whereas two or three intervals prior to the terminal interval less variation allows detection. A choice of dose then should be provided with a time frame to which it applies.

The sponsor's conclusions for the four mash studies are summarized below as choice of dose in grams per ton of Natamycin as determined on the basis of O_2 consumption and CO_2 production. The choices are based loosely on the LSD tests.

Choice of dose by sponsor's analysis

	A. para. 1	A. para. 2	F.moniliforme	P.rubrum
O_2	10	10	5	10
CO_2	5	15	5	none

In dose titration studies, we do not rely solely on tests comparing means of treatment groups. Modeling the dose response curve is our preferred method of determining dose. We fit various models to the oxygen consumption data using the terminal observations for the second Aspergillus study (16 days) and for the Penicillium study (14 days) and the third from the last interval for the Fusarium study (13 days). For each mold, simple linear regressions, indicating increasing effect through the highest concentration were among the top contenders for choice of model. The highest adjusted r^2 values for the Aspergillus data and the Fusarium data came from the linear plateau model III-4. This model predicts decreasing O_2 consumption to 15 grams per ton and then a plateau, indicating no further effect by adding more Natamycin. The adjusted r^2 value for Aspergillus is 0.6630 and for Fusarium is 0.5167. For the Penicillium data the highest adjusted r^2 is 0.4703 for the model III-3, which plateaus at 10 grams per ton. However, the adjusted r^2 for the model III-4 is 0.4655 so they are virtually indistinguishable. Thus, our analysis leads to a recommendation of at least 15 grams per ton rather than 10 grams per ton as chosen by the sponsor.

In the study in pellets, the daily mold growth scores ranged from 0, none seen at 10x or 20x magnification, to 3, massive hyphal development with abundant aerial mycelium and reproductive structures present. By the fifth day, all four of the control plates scored 3 and remained at 3 for the remaining observations at days 6 and 7. In contrast, the average scores at 5, 10, and 20 grams per ton were 2, 1.5, and 1.25 on day 5 and 3, 2, and 1.5 on day 7. This indicates a dose dependent mold retardant effect in pellets.

In summary, we agree that there is a dose dependent mold retardant effect of Natamycin. Modeling leads us to choose a dose of 15 grams a ton. This is higher than the 10 grams per ton, the sponsor has chosen.

II. FIELD TRIALS:

A. Study Design:

The field studies are divided into two subsets. In the first subset, there are studies conducted in Maryland, Louisiana, and Georgia using the same protocols. At each location two farms were selected. One farm was delivered control feed and the other received Natamycin-treated feed from the time the chicks were placed until they were marketed, beginning with starter feed, then grower ration, and finally withdrawal formulation. At each farm three replicate houses were used.

The second subset is one larger study conducted in Georgia. Forty farms were randomly assigned to receive either control feed or Natamycin-treated feed. The number of houses treated at a farm varied from one to six, but the information collected was reduced down to being on a per farm basis regardless of the number of houses used.

In the first subset, samples were collected at the three feed mills while the feed was being produced as mash and then after it was pelleted and prepared as crumbles. Four batches were made at each mill. This was to determine if Natamycin could be added to feed to consistently obtain the appropriate concentration. At each farm, the feeder lines were sampled, Beginning (B), Middle (M), and End (E) in each house. This was done on days 0, and about days 15, 30, and 45. This was to determine if Natamycin segregated or separated from the feed in the feed line. Concentrations of Natamycin were determined by liquid chromatography (HPLC).

House samples (B, M, E) were aggregated and triplicate mold spore counts were taken for each house. Moisture content was evaluated and water was added to increase the moisture contents. Six replicates were formed by placing aliquots of the hydrated feed into flasks. Then the molds were grown in the flasks which were hooked up to the respirometer, similar to what was done in the dose titration studies. After the growth period, mold spore counts were taken for each replicate. The molds were identified. Concentrations of aflatoxins were determined. Production parameters for the 3 houses combined were recorded. These included how many birds were sold and how many were started, their average weights and the feed conversion values.

In the single larger study, the feed mill assays were not included. Farm feed samples were collected on days 0, 30, and 45 but not on day 15. Otherwise, similar information was gathered in this subset as was gathered in the first subset of field trials. One additional feature was added. Farm samples were divided and one subsample was inoculated with a mix of mold spores (*A. parasiticus, P. rubrum,* and *F. moniliforme)* and attached to the respirometer while the mold grew. Mold spore counts were determined at the end of the growth period.

B. Statistical Review:

<u>First subset (three small studies at different locations)</u>

None of the analyses discussed below for the first subset, which included three geographic locations, were done for the three locations combined. A combined analysis should be done to demonstrate that, taking different situations into account, the desired effect is present.

None of the control samples had detectable levels of Natamycin. The feed mill data are presented by batch, displaying the replicate sample assay values and their means, standard deviations and coefficients of variation for both the mash and the crumbles. This is presented to convince the reader that the Natamycin content can be well-controlled. Several values are tossed out in the calculations. In several instances this is because the first samples were not true feed but rather "flush out" material, mostly corn. This should have been detected at the time of sampling and later samples should have been collected. Other values were tossed out because they were abnormally high and it was later decided that this happened because the Natamycin was added simultaneously with liquid ingredients and clumped. See page 1444 (Maryland, crumbles, batch #2). This indicates that mixing instructions

should make it clear that Natamycin should not be added along with the liquid components of feed. Finally, the target concentration was 11 ppm or 10 grams per ton. It is noted that, in general, all the feed tested was low in Natamycin, but crumbled pellets were further below target than mash was. Crumbled pellets contained about 8 ppm across the three locations. The Maryland mill was particularly below target - in the neighborhood of 5.9 to 8.3 ppm in mash.

Analysis of variance was used to analyze the B, M, and E concentrations of Natamycin. The B, M, and E observations from the same house were treated as though they were independent; this does not seem an appropriate assumption here. We treated the B, M, and E samples as repeated measures from the house. And because different types of feed were sampled on different days (mash versus pellets) and these have different concentrations even at the mill where they are made, we analyzed days separately. We did the analysis combining the three locations. We found, first of all, that place in the feed line and location of the field trial do not interact. Thus, we can make a general statement about the effect of place in the feed line. Natamycin concentrations in the feed do not differ by the place in the feed line from which they were sampled. Hence, Natamycin does not segregate nor separate from the feed.

Mold spore counts were analyzed by one-way analysis of variance. Eight treatment groups were formed by combinations of Natamycin concentration and day of sample. The analysis makes no sense to this reviewer because, as in the B, M, and E analysis, the effect of chicken house is ignored. The sponsor's analysis finds no difference between control and Natamycin at any of the three locations. They note how variable the data are. An analysis was performed on subsamples "before respirometry" and "after respirometry". The before respirometry values would seem to be more appropriate for determining the field efficacy of Natamycin. No analysis of the respirometry values (CO_2 production or O_2 consumption) is presented.

We analyzed the mold spore counts as repeated measures on the chicken house, combining all three locations. Count data are frequently transformed by taking the logarithm of the counts prior to analysis. This is considered a variance stabilizing transformation. We used the log transform. There is a significant time by location by treatment interaction, which means that a general conclusion is not warranted. Instead one has to look at individual times. In doing so, we find that there are significant interactions between location and treatment on days 15, 30 and 45. Thus, conclusions about the utility of Natamycin to retard mold growth would

appear to have to be made on the individual location basis. There is insufficient power at individual locations since there are only three houses (observations) per location. Therefore these studies do not confirm the utility of Natamycin.

During mold identification, molds other than the three used in the dose titration were found. They are: Rhizopus, Sporothrix, Mucor, Paecilomyces.

Moisture content of the feed was in the 12.5% to 14% range prior to hydration and respirometry. Aflatoxin concentrations were extremely low in both controls and treated samples.

Very small differences in mortality, average weight of marketed birds, and feed conversion were observed. This information is presented in the submission in the three separate locations' reports, but it is tabled together below for convenience.

So that only one table is required for the production parameters, the large Georgia study results are appended and labeled "GA II".

Production Parameters for Natamycin Field Trials

Location	Treatment	# started	% mortality	avg. weight	feed conversion
Maryland	Control	60,000	1.73	5.06	1.96
	Natamycin	90,447	0.88	5.12	2.04
Louisiana	Control	64,800	5.98	3.89	1.79
	Natamycin	64,800	5.15	4.09	1.78
Georgia	Control	79,000	2.01	5.02	1.93
	Natamycin	77,285	2.31	5.13	1.94
GA II	Control	1,010,845	4.68	5.01	1.95
	Natamycin	959,960	3.93	4.96	1.96

Second subset (one large study conducted in Georgia)

Feed mill samples were not taken. The B, M, and E farm samples were composited to yield one value of Natamycin per farm. Natamycin farm # 16 evidently received the incorrect day 0 feed, as no Natamycin was detected. Thus, for day 0, that farm was eliminated from the calculations of average, standard deviation, and

coefficient of variation. The average (coefficient of variation) for each sampling day in the Natamycin farms are: 8.83 (12.23), 8.40 (9.29), and 8.38 (8.59) for days 0, 30, and 45, respectively. The lowest observed concentration was 5.69 ppm and the highest was 10.7. The sponsor concludes that the Natamycin content was uniform. We agree that the content was uniform, although we also note that the feed uniformly does not contain the targeted amount of Natamycin.

For each farm, the average of three replicate mold spore count determinations is listed. Descriptive statistics and a 95% confidence interval on the average (over the 20 farms) spore counts are given for each treatment group on each sampling day. The sponsor does not test for treatment differences, but states that the mold spore plate counts were consistently lower in samples collected from Natamycin farms than those collected from controls farms. These results should be formalized with a statistical test and since the count data are skewed, we used a nonparametric test, the Wilcoxon rank sum test. The day 0 counts are almost significantly lower in the Natamycin group, $p \leq 0.0531$ and are extremely so at day 30, $p \leq 0.0001$, and at day 45, $p \leq 0.0003$.

Similar information is presented for the farm samples after respirometry and for inoculated samples after respirometry. These variables are not relevant for confirmation of efficacy in the field.

Molds identified in this study are the same as those identified in the first subset of field studies.

The average day 0 feed moisture contents were 12.74 in the controls and 12.63 in the treated samples. Day 30 values were around 13.25 and day 45 values were around 13.9, indicating that the feed was picking up moisture over time.

Aflatoxin concentrations were low in the day 0 and day 45 samples, but higher in day 30 samples, with an average of 1.68 ppb in the controls and an average of 1.98 ppb in the treated samples. Individual farm values were quite variable, with 10 control farms and 12 treated farms having no detectable aflatoxins.

Production parameters are summarized in the table above along with the results from the three studies in subset one.

Conclusions from the field studies:

It is evidently difficult to produce the feed such that it has the targeted concentration of Natamycin using the instructions or current procedure. The instructions should be adjusted to increase the Natamycin concentration in the finished feed and should warn against adding Natamycin along with liquid ingredients.

At the levels actually used in the first subset of studies, the utility of Natamycin to retard mold growth was not demonstrated. This may be because the level was insufficient. The studies were small and spore counts were only slightly smaller in the Natamycin group (Maryland and Georgia) or slightly higher (Louisiana).

At the level used in the second subset of studies, the larger number of studies allowed detection of a shift in the distribution of spore counts in favor of the Natamycin treated feed.

Therefore, we have reviewed only one study conducted in Georgia that confirms that Natamycin added to feed, at concentrations assayed to be about 8.5 ppm, retards mold growth relative to that seen in untreated feed. The sponsor has not demonstrated that this effect can be achieved at other locations, as the multiple geographic location study does not demonstrate the utility of Natamycin at the levels used.

Production parameters in chickens eating feed treated with Natamycin are similar to those in chickens eating control feed for the duration of the studies.

III. COMMENTS THAT MAY BE CONVEYED TO THE SPONSOR:

1. Biometrics recommends that in future dose titration studies you model the dose response. The model will indicate the appropriate choice of dose. We modeled the oxygen consumption data for the terminal intervals for Aspergillus and Penicillium and the day 13 values for Fusarium. We chose models that show a decrease in consumption from 0 to 15 grams per ton and then a plateau from 15 to 20 grams per ton as the ones which describe the data best. Based on the models, our recommendation for dose is 15 grams per ton. However, we recognize the inoculation method used represents an extreme challenge.

2. In field studies, in addition to separate analyses of the individual locations conducted under the same protocols, we request that a combined locations analysis be performed to the extent possible. The goal is to demonstrate that the treatment is effective in various settings.

3. The Beginning, Middle, and End samples from one chicken house are more appropriately treated as repeated measures from the house. Using a repeated measures analysis and combining the three studies, we agree that Natamycin does not separate from the feed.

4. We also attempted to conduct a repeated measures analysis on the mold spore counts taken over time from the same house. First we used the log transformation on the counts to stabilize the variance. We found we could not pool the locations for this analysis as there was a significant time by location by treatment interaction. At individual locations, the treatment effect is not significant. The three-geographic locations study does not confirm the utility of Natamycin for retarding the growth of mold in chicken feed at the levels used.

5. We note that the levels actually used were considerably less than the 11 ppm intended.

6. Despite the fact that the assayed levels in the study involving 40 farms in Georgia were also assayed below target at about 8.4 ppm, significant reductions in spore counts were seen. We applied the Wilcoxon rank-sum test to the counts at each time they were determined. The day 30 and day 45 tests were highly significant; the day 0 test was nearly significant.

7. In summary, modeling in the dose titration study indicates that under the extreme circumstances created by inoculation, an optimal choice of dose is 15 grams per ton. Assayed concentrations of Natamycin in your field trials were below the targeted amount of 11 ppm (or 10 grams per ton). One large trial at Georgia demonstrates that, even at about 8.4 ppm, Natamycin has a mold retardant effect. You have not presented evidence that this effect is reproducible at other locations representative of varying conditions.

IV. CONCLUSION:

The statistical methods used in the dose titration study were appropriate as far as they went. In addition to testing among doses, we rely on modeling the dose response to help determine a dose. The models chosen indicate a dose of 15 grams per ton.

The statistical methods used in the confirmation studies at three geographic locations were not appropriate where they ignored the effect of chicken house. No combined analysis of the three geographic locations was presented. The studies were too small and did not support the efficacy of Natamycin.

No statistical test was supplied for the spore count data from the larger Georgia study. A nonparametric test, the Wilcoxon rank-sum test, was used by us and shows the significance of the reduction in mold in the Natamycin-treated feed at days 30 and 45.

Thus, there is only one study at one location that confirms that a concentration of Natamycin actually used (and below 11 ppm) does what it is purported to do.

At the primary reviewer's discretion, we hope to see the firm receive some advice about the mixing instructions. We do not think it is in Biometrics' purview to comment on this subject. We have something like the following in mind. The mixing instructions should be revised so that the process leads to levels closer to the intended concentrations. The instructions should also include a warning to not add the Natamycin at the same time as liquid ingredients are being added to prevent clumping.

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