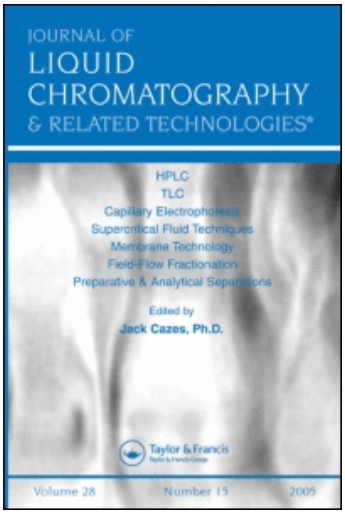

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Derivatization of HPLC/Fluorescence Quantitation of Maduramicin Ammonium in Feed and Premixes at Levels Down to 5 ppm

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DERIVATIZATION AND HPLC/FLOURESCENCE QUANTITATION OF MADURAMICIN AMMONIUM IN FEED AND PREMIXES AT LEVELS DOWN TO 5 PPM

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ABSTRACT

A reverse phase HPLC method with fluorescence detection and pre-column derivatization is described for determination of maduramicin (and possibly other non-fluorigenic ionophores containing a hemiketal ring) in feeds, premixes, and technical material. Dansyl hydrazine derivatization together with Florisil Sep-Pak clean-up is described. Isocratic HPLC with external standard is used. Method development, specificity and the chemistry of derivatization is discussed.

INTRODUCTION

Ionophores are biologically active carboxylic acid polyethers. Commercially significant are Monensin (I), Lasalocid (II) and Maduramicin (III) which are used in the treatment and prevention of coccidiosis in poultry. Normal administration mode is by ingestion from a premix-medicated poultry feed. A premix is

a finely divided solid containing active ingredient at relatively low concentration and is designed for mixing with feeds at the rate of 0.5-10 kilos/ton.

Maduramicin is a potent coccidiostat and treatment level in feed for poultry use, is only 5 ppm active material. An HPLC analytical procedure was required to quantitate the active material in the feed at this level. Because the method of Johnson[1] was considered too exotic for general use and because Maduramicin lacks appreciable U.V. and electrochemical properties the fluorigenic labeling technique of Frei and Lawrence[2] was investigated in order to achieve nanogram level quantitation without pre-concentration. Their technique is used for the derivatization of carbonyl compounds with dansyl hydrazine to form the dansyl hydrazone in the presence of acid-catalyst and heat.

The Maduramicin molecule contains a hemiketal at both the A and F rings and acid hydrolysis could generate the carbonyl radical and hence the dansyl derivative somewhat analogous to the reaction of glucose with phenyl hydrazine to form the phenyl hydrazone.

The Frei and Lawrence procedure was modified to produce the dansyl derivative almost instantaneously without the use of heat and without an evaporation step (in order to quench the reaction).

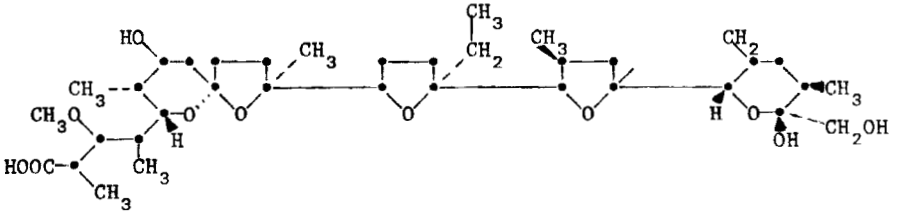
This paper deals with the development, validation and chemistry of the derivatization of maduramicin for fluorescence detection by HPLC quantitation in feeds, premixes and technical material. With modification, the procedures are applicable to Monensin (I) and Nigericin (IV) and other molecules and ionophores containing a cyclic hemiketal or reactive carbonyl group. The quenching for these needs to be resolved.

EXPERIMENTAL

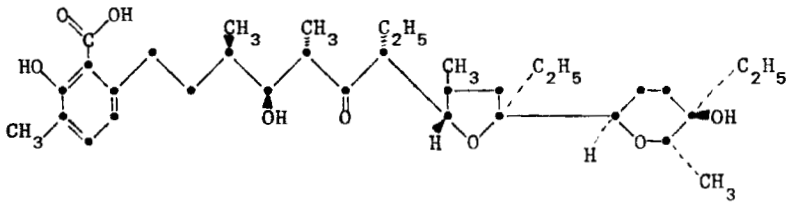
Reagents

Maduramicin analytical standard from American Cyanamid Company (Princeton, NJ); dansyl hydrazine and tetrabutylammonium

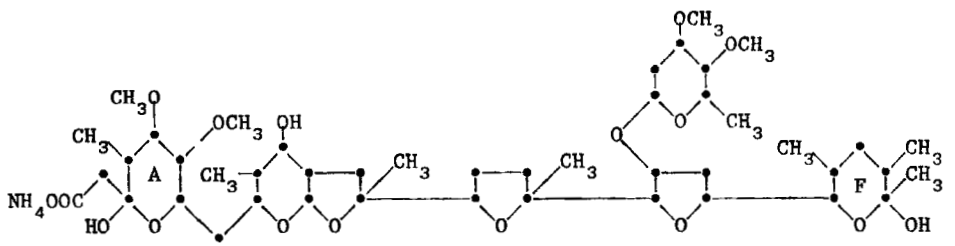
Commercial Polyethers



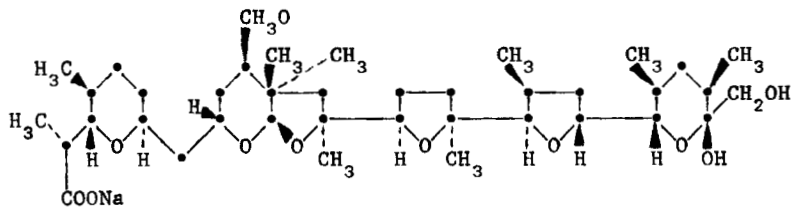
MONENSIN (I)



LASALOCID (II)



MADURAMICIN (III)



NIGERICIN, SODIUM (IV)

hydrogen sulfate from Regis Chemical Company (Morton Grove, IL); acetonitrile, HPLC grade from J. T. Baker Chemical Company (Phillipsburg, NJ); Florisil Sep-Pak Cartridges from Waters Associates (Millford, MA); and deionized water was put through a Milli-Q Ultrapure Water System from Millipore Corporation (Bedford, MA). All other chemicals were reagent grade.

Apparatus

The fluorescence detector was a model FS970 spectrofluoromonitor and was equipped with optional automatic overload reset (from Kratos Instrument Company, Ramsey, NJ). The liquid chromatograph consisted of an isocratic DuPont 870 triple piston pump, a Varian 8000 Autosampler, a Valco ACGW Valve with 60 µL loop and an IBM 5 µm C_{18} Mini Column (5 cm x 4.6 mm) held at ambient temperature.

Standard and Recovery Solutions

Stock solution of maduramicin at 1 mg/mL was prepared in HPLC grade acetonitrile. This was diluted to 50 mcg/mL and further diluted to 1-3 mcg/mL. Spiking was not made directly onto feed, but into the appropriate volume of extraction solvent (acetonitrile) covering the feed.

Liquid Chromatography

Isocratic conditions with an IBM C-18, 50 mm standard column and 25/75 water/acetonitrile modified with 0.25 g tetrabutylammonium hydrogen sulfate per liter (25/75) at a flow rate of 2.0 mL/min are used (pH ~3.5). Quantitation is performed at 1-3 mcg/mL using 60 mcL loop and fluorescence detector using an

excitation wavelength of 210 nm and 320 nm cutoff filter at the photomultiplier tube.

Analyte response is measured by peak height of the major peak which elutes last while the minor peak is not stability indicating. Related recoveries and assays are calculated by comparison to external standard response. Standard injections bracket every 3-5 sample injections, to compensate for any response changes during multiple sample runs.

Sample Preparation

Technical material is dissolved in acetonitrile to contain about 1 mg/mL and diluted to approximately 3 mcg/mL for derivatization and quantitation.

Premix (usuallly 1% AI) is likewise diluted to 3 mcg/mL where 0.25 g premix is a satisfactory amount to dissolve in acetonitrile. Sonic bath is used to ensure dissolution.

Feeds at 5 ppm are extracted with acetonitrile to produce an extract containing 1 mcg/mL (50 g/250 mL). Thirty minute shaking is required for extractions. Feed recoveries using blank feed plus 245 mL acetonitrile and a 5 mL spike of standard solution (50 mcg/mL) are treated in a similar manner.

Derivatization

1. For Premixes and Technical Materials

Transfer, in the order below, the following ingredients to a 1 mL nominal Wheaton GC vial: 30 mg (±3 mg) calcium carbonate; 100 mcL dansyl hydrazine (7.5 mg/mL in ACTN); 1.0 mL of sample or standard solution (3 mcg/mL); 100 mcL trichloroacetic acid (150 mg/mL in acetonitrile).

Cap the vial and shake vigorously by hand for exactly 60 seconds. Centrifuge immediately at high speed for 1 minute. Inject immediately into HPLC (response not stable for more than 10 minutes). A C.V. of 0.83% can be expected if these conditions are met.

Premix and technical samples in acetonitrile, derivatized by the above procedure should be compared to standards of similar concentration derivatized by the procedure given above. For Monensin and Nigericin, do not use calcium carbonate, and use greater ratio of acetonitrile in mobile phase.

2. For Feed Samples

Transfer 0.6 mL each of dansyl hydrazine and of trichloroacetic acid (concentration as above) to a 5 mL reaction vial (Teflon liner in cap). Transfer 2 mL of clarified sample extract, cap vial and shake by hand for 10 seconds. Put vial contents through a Florisil Sep-Pak, dropwise at approximately 1 drop/second ($\pm$ 0.25 drops/second).

Wash Sep-Pak with 3 x 5 mL aliquots of acetonitrile (1 drop/second). Purge Sep-Pak with air to remove occluded solvent. Elute Sep-Pak with 10% water in acetonitrile collecting 5 mL of eluate. Mix eluate and transfer to autosampler vial for injection (response for this procedure is stable for 24 hours). All feed samples should be compared to standards derivatized in similar manner. For Monensin and Nigericin, Sep-Pak clean-up and quenching has not been optimized so that little, if any response is obtained if quenched in this manner.

RESULTS AND DISCUSSION

1. Derivatization and Quenching

The procedure of Frei and Lawrence for the derivatization of carbonyl compounds with dansyl hydrazine in the presence of glacial acetic acid, requires heating to 70°C for approximately 15 minutes. The acetic acid is evaporated off on a Rinco to quench the reaction and the derivative is usually dissolved in toluene for normal phase HPLC injection.

Because the monitoring HPLC system of choice then, was a reversed phase system which Barbatschi[3] had developed with acetonitrile/phosphate buffer (pH 6.5) as mobile phase and U.V. detection at 254 nm the Maduramicin reaction mixture (in acetonitrile) was cooled down to RT and quenched by direct injection into the HPLC system. Remarkably, the first such test showed clearly the presence of a derivative peak since a reagent blank similarly treated, lacked this peak. Longer reaction times produced a larger response and secondly, the appearance of an earlier eluting minor peak. The switch to trichloroacetic acid catalyst produced major and minor peak even when heating time was reduced to zero. Clearly, heating is not essential for derivatization when catalyzed by trichloroacetic acid.

Linearity of response was evident even under the crude conditions above and improved after catalyst/reagent and reagent/sample optimization was effected. Excellent reproducibility and linearity were obtained by partial reaction quenching with calcium carbonate powder (good for about 10 minutes) or complete quenching by passing the reaction mixture through a Sep-Pak Florisil column before injection (good for 24 hours).

A loaner fluorescence detector from Kratos (FS970) revealed the full sensitivity protential of the

simplifications (modifications) above. With excitation wavelength at 210 nm excellent linearity of response ranged between 0.2 to 10.0 mcg/mL with a correlation coefficient of 0.99988 for derivatization followed by Sep-Pak clean-up. Other excitation wavelengths (260, 350 nm maxima) give lower (~50%) response than at 210 nm.

2. Specificity

A radio-tagged study using hot and cold Maduramicin and HPLC with fluorescence and radio flow monitors in series, showed that the ratio of the minor to the major peak is about equal by both detection systems with approximately a 40/60 distribution between peaks.

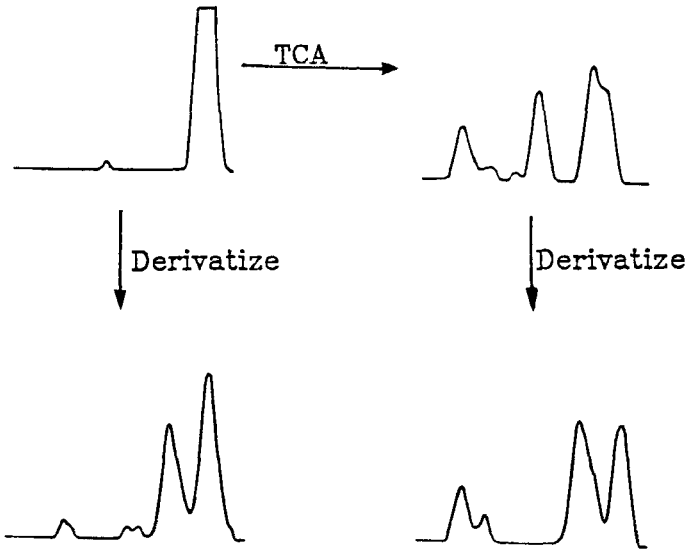


FIGURE 1. Radio monitor chromatograms.

Acid hydrolysis with trichloroacetic acid (TCA) shows degradation of parent which when followed by reaction with dansyl hydrazine, results in a decreased major peak indicating method specificity for parent. The minor peak does not always reflect parent degradation and is not used in quantitation.

Intact parent whether it be in the sodium or free acid form, or its F-ring methyl ketal all show the same retention time as Maduramicin (the ammonium salt) and are not differentiated from parent. All other transformations of the molecule show altered retention time (e.g. decarboxylated A-ring, dehydrated B- or F-ring or combinations). The beta-isomer elutes at approximately 1/3 the retention of parent.

Cyanamid's Gosport Laboratory has shown the feed method viable in the presence of 26 coccidiostats and antibiotics. (below)

Assay of Animal Feed Additives in Blank Broiler Ration*

Additive	Inclusion Level (PPM)	Assay Equivalent (PPM)
Flavomycin	10	Less than 0.5
Virginiamycin	10	"
Nitrovin	10	"
Furazolidone	10	"
Nifursol	50	"
Tylosin	100	"
Avoparcin	10	"
Chlorotetracycline	165	"
Sulphadimidine	165	"
Procaine Penicillin	82.5	"
Zinc Bacitracin	100	"
Olaquindox	75	"
Sulphaquinoxaline	20	"
Pyrimethamine	10	"
Carbadox	75	"

Note: Detection limit of Maduramicin in feed is 0.5 ppm.
*Cyanamid U.K. Data.

Assay of Coccidiostats in Blank Finisher Ration*

Coccidiostat	Inclusion Level (PPM)	Assay Equivalent (PPM)
Salinomycin	60	Less than 0.5
Monensin	100	"
Lasalocid	90	"
Narasin	70	"
Stenerol	3	"
Amprolium	125	"
Ethopobate	8	"
Clopidol	125	"
Robenidine	33	"
Arprinocid	60	"
Methyl Benzoquate	100	"

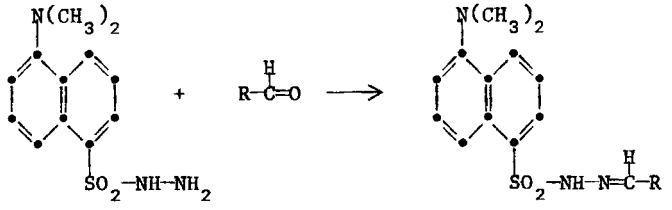
Note: Limit of detection of Maduramicin in feed is 0.5 ppm.
*Cyanamid U.K. Data.

The method has been successfully used to assay for III in feeds originating from a large number of sources, including Brazil, Spain, South Africa, Israel and Great Britain.

3. Chemistry of Derivatization

Dansyl hydrazine reacts with the carbonyl group to produce the dansyl hydrazine derivative.

Chemistry of Derivatization

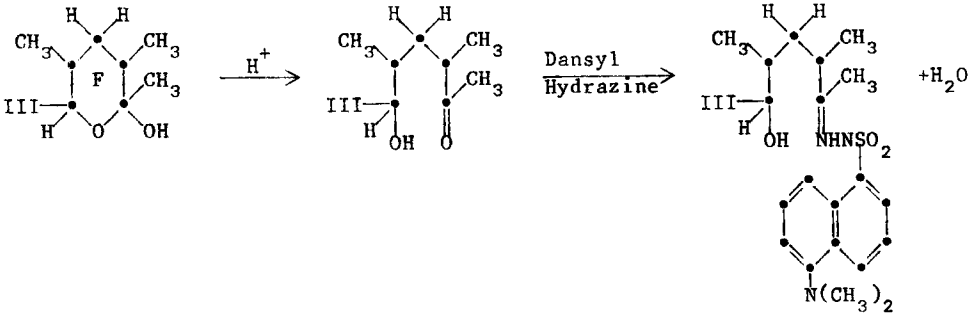


Although the Maduramicin molecule (III) exhibits no carbonyl as such, the A- and F-rings are cyclic hemiketals

and produce the free carbonyl (below) under acidic conditions.

The major peak is postulated to follow the reaction route below through the F ketal ring:

Postulated Reaction Mechanism for Major Peak



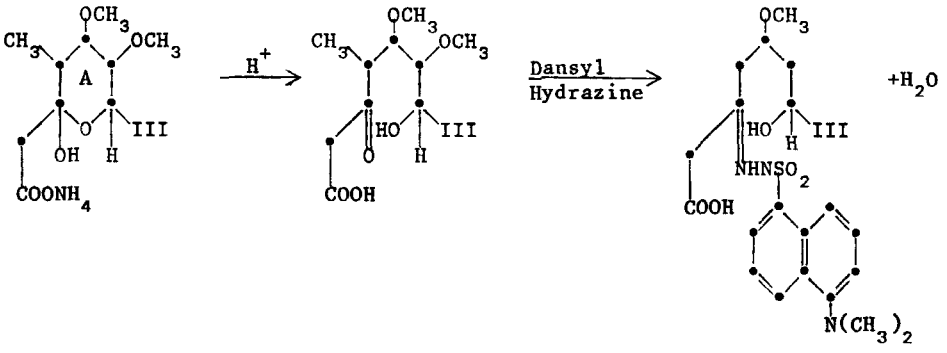
The identity of the derivative is supported by the M.W. (1186 daltons) obtained by desorption mass spectrometry performed on the peak after isolation by HPLC, and by the absence of a derivative when the ketal is absent (e.g. lasalocid does not give a derivative nor does dehydrated F-ring).

Similarly, the minor peak is postulated to be the hydrazine derivative of the A-ring ketal since its M.W. is also 1186 daltons and ionophores with only one ketal (Monensin, Nigericin) give only one derivative peak in the HPLC, whereas Maduramicin, with two ketal rings, gives two derivatives. The generation of two peaks also occurs with related (Maduramicin) compounds which contain two ketal rings.

4. Recoveries

Six recoveries on each of three separate days were run, as described under "Standard and Recovery Solutions," on

Postulated Reaction Mechanism for Minor Peak



Maduramicin Recoveries from Feed at 3-11 PPM

	PPM theory (nominal)	Day 1 %	Day 2 %	Day 3 %
1	3	83.3	96.8	99.7
2	3	103.1	74.8	92.7
3	5	94.4	97.7	93.9
4	5	103.0	92.0	95.2
5	10	110.0	102.2	105.6
6	10	112.9	97.8	108.5
Mean		101.1	97.3	99.3
Std Dev		10.9	10.6	6.5

feeds over the nominal range 3, 5 and 10 ppm and processed as for sample. Recoveries ranged between 74.8 and 112.9% with mean of 98.0% and coefficient of variation of 9.2%.

These statistics agree with those to be expected[4] at ppm analyte concentrations. In a single experiment, feeds which were dry-spiked with premix (1:2000 dilution) yielded an average recovery of 102% and agreed well within experimental error. Recovery from standard spiking solution onto dry feed was 99% (immediate extraction after spiking) and 95% (extracted 1 hour after spiking, duplicate determinations).

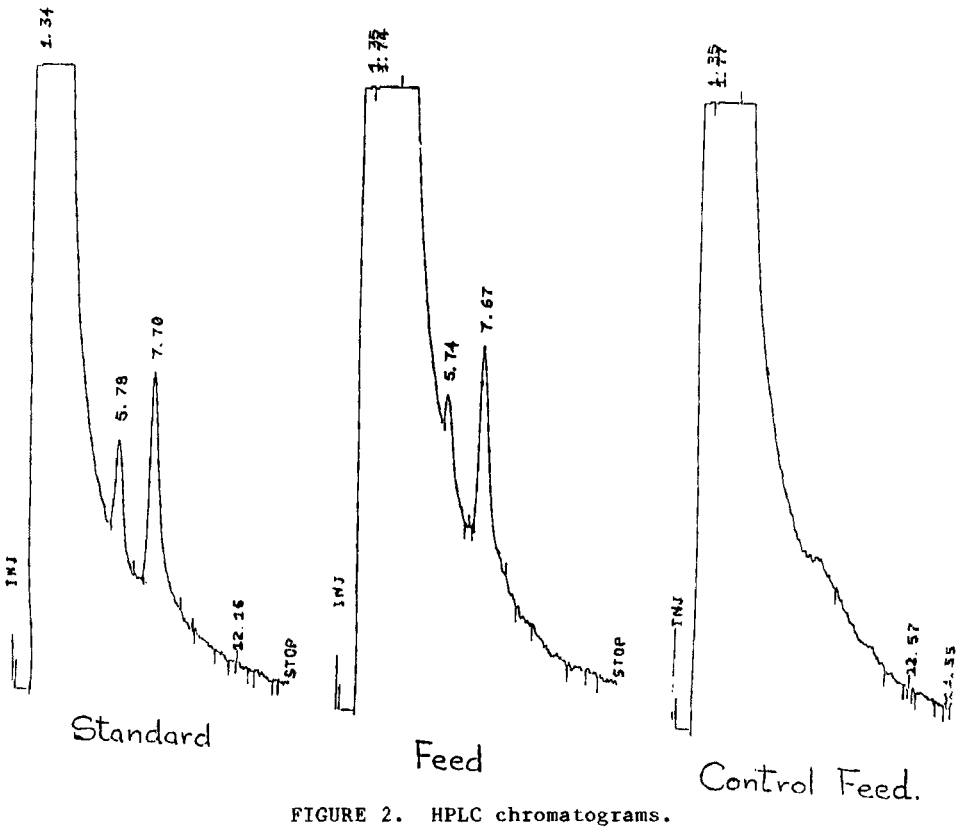


FIGURE 2. HPLC chromatograms.

No technical or premix recoveries have been run to date. However, the method described above has always given excellent agreement with the premix method (refractive index detection), which has been subjected to a rigorous validation study. Typical chromatograms for the feed method are shown below.

ACKNOWLEDGEMENTS

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Barbatschi for his HPLC system, Bob Maxwell ex-Kratos for a loaner FS970 fluorescence detector and W. C. Groth for his faith and confidence assigning this problem to me.

REFERENCES

1. Johnson, N. A., The Quantitation of Maduramicin, Monensin, Nigericin and Lasalocid at the Nanogram Leval using HPLC Atomic Absorption Spectroscopy. Paper No 1130, Pittsburgh Conference, 1985.

2. Frei, R. W. and Lawrence, J. F., Fluorigenic Labeling in High-Speed Liquid Chromatography. Journal of Chromatography, Vol 83, 321, 1973.

3. Barbatschi, F., Interim HPLC Process Control Procedure for Maduramicin. Private Communication.

4. Horwitz, W., Kamps, L. R. and Boyer, K. W., Quality Assurance in the Analysis of Foods for Trace Constituents. J. Assoc. Off. Anal. Chem., Vol 63, No. 6, 1980.