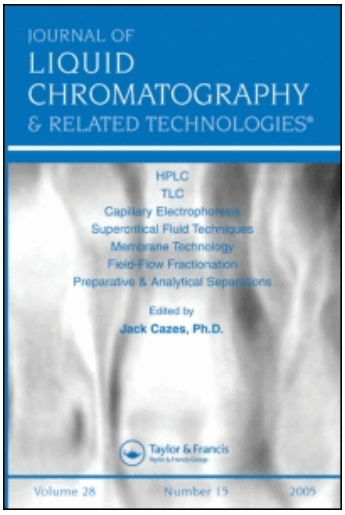

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF VIRGINIAMYCIN IN PREMIXES AND FEEDS

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ABSTRACT

A simple and practical method for the determination of virginiamycin (VGM) in premixes and feeds is described. VGM in premixes and feeds is extracted with methanol, and subsequently analysed by high performance liquid chromatography (HPLC) with ultraviolet detector set a wavelength at 235 nm. The column is Kaseisorb LC ODS-300-5 and the eluent is acetonitrile-water (35 : 65, v/v). The VGM concentration in various samples is calculated from the peak hight of virginiamycin-M_1 (M_1), of main component of VGM. Amounts of 0.2-20.0 μg/ml of VGM can be determined. The detection limit for VGM is 2.0 ng. The average recoveries were 93.3 % and 86.6 % for VGM added to feeds at the level of 20μg/g and 10μg/g, respectively.

INTRODUCTION

VGM is one of the streptogramin family of antibiotics. VGM is a mixture of two main active components consists of factor M (M_1 and M_2) and S (S_1,S_2,S_3,S_4, and S_5), and generally used as a feed additive for poultry.

Based on the previous findings(1) that biological activity of the crude VGM was almost attributable to M_1 which is a main component of VGM, the measurement of M_1 might make it possible to develop the simple and rapid analysis of VGM in premixes and feeds. The rapid and simple method for the routine analysis of VGM is required in respect with the quality assessment of premixes and feeds and health concern.

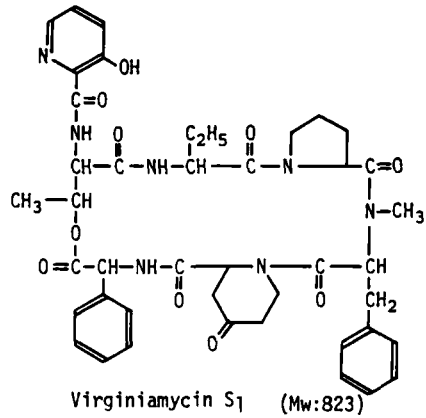
For the determination of VGM, several methods such as turbidimetrically(2) and biologically(3-5) have been proposed. However, VGM is usually determined by a biological method based on the measurement of total antibacterial activity. These biological methods were not suitable for routine analysis because of the interferents from other antibiotics or active degradation products and time consuming.

This paper proposes an accurate HPLC method for the determination of VGM in premixes and feeds by measuring M_1.

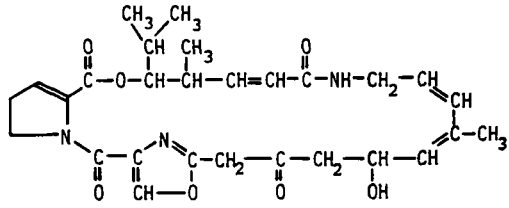
MATERIALS AND METHODS

Reagents and apparatus

VGM was supplied from Nihon Zenyaku Kogyo (Tokyo, Japan). A stock solution of VGM was prepared by dissolving VGM in methanol to give a concentration of 1000 μg/ml(reference standard had a potency of 190 % according to supplier). A series of working standard VGM solution were prepared by diluting the stock solutions



Virginiamycin S_1 (Mw:823)



Virginiamycin M_1 (Mw:525)

FIGURE 1. Structures of virginiamycin M_1 and S_1.

with methanol to appropriate concentration. Commercial premix products as STAFAC 20 and STAFAC 500 were supplied from Nihon Zenyaku Kogyo. M_1 was prepared from VGM by the method reported previously(1). Acetonitrile and methanol were of HPLC grade (Wako Pure Chemical Industries, Japan). All other chemicals were of reagent grade and used without further purification. Water used was distilled and deionized.

The HPLC was carried out using a Shimadzu LC-6A system (Shimadzu Seisakusho, Japan) equipped with a SPD-6A spectrophotometer set a wavelength at 235 nm. The column used was kaseisorb LC ODS-300-5 reversed phase column(4.6 mm I.D. × 250mm, Tokyo Kasei Kogyo, Japan). The HPLC mobile phase was a mixture of

acetonitrile and water (35 : 65, v/v). The mobile phase was run isocratically at ambient temperature at a flow rate of 0.7 ml/min.

Calibration graph

A 20μl of working standard VGM solution in the range of 0.2 to 20.0 μg/ml was injected into the HPLC. A calibration graph was constructed by plotting the peak height of M_1 versus the concentration of VGM.

Sample preparation and their determination

(a) Determination of VGM in premixes

About one hundred mg of premix sample was accurately weighed into a 100 ml volumetric flask. A 50 ml of methanol was added and the mixture was extracted for 5 min in an ultra sonic bath. The contents of the volumetric flask were then filtered. The filtrate was diluted with methanol so as to obtain the VGM concentration containing ca. 10 μg/ml. An aliquot of the final solution was injected directly into the HPLC as in described procedure. The VGM concentration in samples was determined by measuring of the peak height of M_1.

(b) Determination of VGM in feed

About 5 g of the feed sample was weighed into a 200 ml glass-stopped flask, and 70 ml of methanol was added. The mixture was shaken mechanically for 10 min. The contents of the flask were then filtered, and the residue was rinsed with an adequate volume of methanol. These filtrate were combined, and then made the volume to 100 ml with methanol. A 5 ml of aliquot was evaporated to dryness, and dissolve in 1 ml of the mobile phase. The solution was filtered with millipore filter (0.5μm), if necessary.

(C) Microbiological assay of premixes

The antibacterial activity of VGM in premixes was determined by a paper disk method with Micrococcus luteus ATCC 9341 as a test organism. The assay procedure was carried out according to the method of Ragheb(4).

RESULTS AND DISCUSSION

Conditions for HPLC

As shown in Figure 2, the maximum absorption of pure M_1 was found to occur at 223 nm. However, when feed was subjected to the HPLC set at 223 nm, the base line of the chromatogram was drifted,

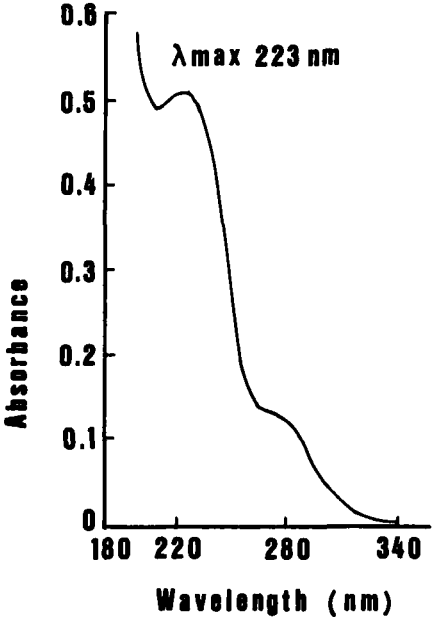


FIGURE 2. Ultra violet absorption spectrum of M_1 in mobile phase.

and the measurement of M_1 peak was interfered with unknown peaks derived from the sample matrix. On the other hand, when wavelength 235 nm was used, the M_1 peak was well defined with respect to the baseline. Therefore, in practice 235 nm was chosen.

In order to find out which column was most suitable in the chromatographic system, several columns such as Kaseisorb LC ODS-300-5, Nucleosil 5 C_{18}(Nagel, FRG), Partisil 5 ODS-3(Whatman, USA), Lichrosorb RP-18(Merck, FRG), μ-Bondapack 18(Waters, USA), and Zorbax ODS(Duppon, USA) were studied with elution solvent as described below. The good peak shape and high resoluton of M_1 was obtained with Kaseisorb LC ODS-300-5 within the studied columns.

Increasing the concentration of acetonitrile in water reduced the capacity factor of M_1. No potentially interfering peaks were observed in the chromatograms when M_1 was eluted with 35 % acetonitrile in water. The chromatograms of M_1 derived from premixes and feed are given in Figure 3. A simple extraction with methanol was sufficient to separate M_1 from the insoluble premix or feed matrix.

Calibration graph

The relationship between the peak height of M_1 in VGM standard solution injected was rectiliniear from 0.2 to 20.0 μg/ml of VGM. The wide range sample concentration required the several signal attenuations during the testing. The reproducibility of the method was examined by analysing samples whose concentrations of VGM were 0.2, 2.0, and 20.0μg/ml (N=5). The corresponding coefficients of variation were 7.7, 2.2, and 0.5%, respectively.

Interferences

The interference of other antibiotics used for feed additives on the HPLC chromatogram was studied using tetracycline,

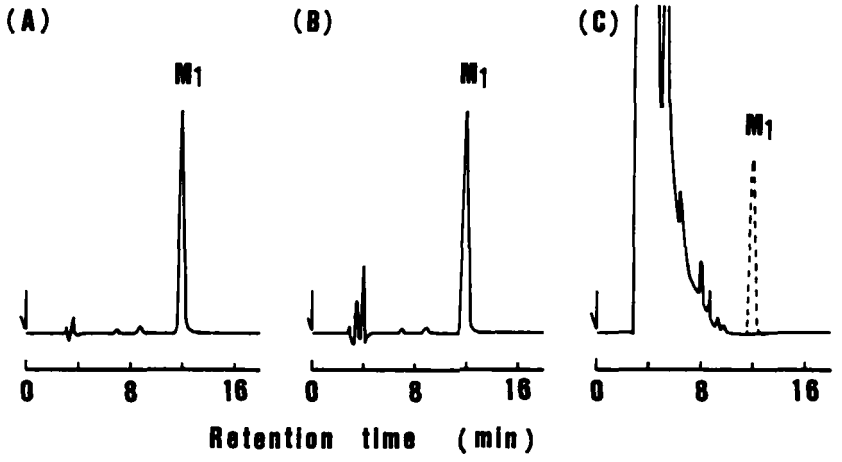


FIGURE 3. Liquid chromatograms of premixes and feed. A : STAFAC 500, B : STAFAC 20, C : poultry feed ; dotted line : sample added VGM to 20μg/g ; conditions as described in the text.

chlortetracycline, oxytetracycline, erythromycin, macarbomicin, oleandmycin, spiramycin, tylosin, monensin, salinomycin, lasalocid, colistin, chroramphenicol, thiamphenicol, and bicozamycin. No interference was observed on the detection of M_1 peak.

Recovery Study and Application of Commercial Premixes

VGM spiked with 10μg/ml and 20 μg/ml were analysed using the the previously mentioned procedure. The average recoveries were 86.6 % and 93.3 %, with high coefficient of variation of 3.7 % and 7.8 %, respectively.

In order to compare the proposed method with bioassay for the determination of VGM in premixes, sample solutions obtained from treating premixes such as STAFAC 20 and STAFAC 500 were applied for two method. As demonstrated in Table 1, the results obtained from the proposed method were in fair agreement with those of bioassay.

TABLE 1

Virginiamycin Contents in Commercial Premixes by HPLC or Bioassay Method

Method	STAFAC 500	STAFAC 20
	(μ g/mg)	
HPLC	474.1	20.2
Bioassay	466.9	19.2

CONCLUSION

In this study, VGM can be determined by measurement of M_1 using HPLC after sample extraction with methanol without a cleanup step. The method is simple, which permits an analysis to be completed within 30 min. It has successfully been applied to the determination of VGM in premixes and feeds. Consequently, these results indicate that the quantitative analysis of VGM can be evaluated in terms of quality assessment of VGM-fortified feeds and premixes. Furthermore, the authors are currently attempting to extend the application of HPLC determination method to residual analysis of VGM in livestock products.

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