Journal of Chromatography, 454 (1988) 387–391
Elsevier Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 784

Note

Isolation of virginiamycin-M_1 by droplet counter-current chromatography

KOICHI SAITO*, MASAKAZU HORIE, YOUJI HOSHINO and NORIHIDE NOSE
Saitama Institute of Public Health, 639-1, Kamiokubo, Urawa-shi, Saitama 338 (Japan)
YASUO SHIDA
Tokyo College of Pharmacy, 1432-1, Horinouchi, Hachioji-shi, Tokyo 192-03 (Japan)
and
HIROYUKI NAKAZAWA and MASAHIKO FUJITA
The National Institute of Public Health, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108 (Japan)
(Received June 27th, 1988)

Droplet counter-currrent chromatography (DCCC) is a preparative separation technique based on the partitioning of a solute steady stream of droplets of mobile phase and the surrounding stationary phase. It is particularly useful for polar compounds and has been applied to the isolation of various types of natural products[1–3].

Virginiamycin (VGM), which is generally used as a feed additive for poultry, consists of factor M comprising M_1 and M_2 and factor S comprising S_1, S_2, S_3, S_4 and S_5. Since it is expected to have determined M_1, which is the main component of VGM, in rapid and simple routine analysis of VGM, the pure compound which is not commercially available, is required.

For the isolation of pure M_1 from crude VGM, Vanderhaeghe *et al.*[4] used silica gel open-column chromatography. However their method did not give sufficient separation of M_1 and M_2 from the crude VGM. The isolation and purification of M_1 from crude VGM by means of DCCC and, furthermore the correlation between the antibacterial activity of M_1 and VGM against Gram positive bacteria were investigated.

EXPERIMENTAL

Materials and apparatus

The crude VGM was obtained from Nihon Zenyaku Kogyo (Tokyo, Japan). All other reagents and solvents with high purity were obtained from Wako Pure Chemical Industries (Osaka, Japan) and were used without further purification. The silica gel pre-coated plate used for thin-layer chromatography (TLC) was Kieselgel 60 of 1 mm thickness and with a fluorescence indicator (Merck, Darmstadt, F.R.G.). The culture medium used for bioassay was antibiotic medium 11 (Difco, U.S.A.). All water used was distilled and deionized.

0021-9673/88/$03.50 © 1988 Elsevier Science Publishers B.V.

The DCCC chromatograph used for separation of M_1 from crude VGM was supplied by Tokyo Rikakikai (Tokyo, Japan). The column consists of a hundred glass tubes (400 m × 3.4 mm I.D.) interconnected in series by capillary PTFE tubes. The samples were dissolved in a mixture of equivalent volumes of mobile and stationary phase and subsequently injected for DCCC using a 5-ml sample chamber. The mobile phase flow-rate was 24 ml/h and the eluate was collected in 5-ml fractions with a fraction collector (SF-160K; Toyo, Tokyo, Japan). The fractions eluted from the columns were monitored by an UV spectrophotometer (330; Hitachi, Japan) at 300 nm. The mixture of benzene–chloroform–methanol–water (26:14:24:6) used as the solvent system was allowed to equilibrate in a separatory funnel. Each column in DCCC had a lower layer of the stationary phase and an upper layer of the mobile phase.

For identification of M_1, an M-80 mass spectrometer (Hitachi, Japan) equipped with an M-003 data processing system was used. The ionization energies and trap currents were 70 eV and 60 μA, and the accelerating voltage was 3.0 kV for electron-impact mass spectrometry (EI-MS). For chemical ionization mass spectrometry (CI-MS), isobutane was used as the reagent gas.

An infrared absorption spectrophotometer (IR-435; Shimadzu Seisakusho, Japan) was also used for identification of M_1.

High-performance liquid chromatography (HPLC) was carried out using an LC-6A system (Shimadzu Seisakusho) equipped with an SPD-6A spectrophotometer set at a wavelength at 223 nm and a RF-530 fluorescence detector set at an excitation wavelength of 314 nm and an emission wavelength of 425 nm. A Kaseisorb LC ODS-300-5 reversed-phase column (250 mm × 4.6 mm I.D.; Tokyo Kasei Kogyo, Japan) was used. The mobile phase was acetonitrile 0.01% phosphoric acid solution (35:65) and chromatography was performed isocratically at ambient temperature at a flow-rate of 0.7 ml/min.

Isolation and purification of M_1

A 50-mg amount of crude VGM dissolved in the mixture of two phases was subjected to DCCC under the conditions described above. The chromatogram is shown in Fig. 1. Fractions of 450 to 750 ml eluted were evaporated at 40°C under reduced pressure. The residue was dissolved in an adequate volume of chloroform, and the mixture was spotted on a preparative TLC plate. Development was with the solvent system chloroform–methanol (95:5, v/v) in an equilibrated tank at room

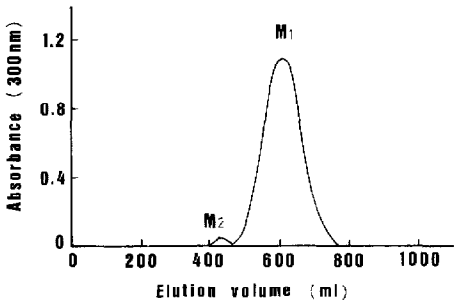

Fig. 1. Elution profile of virginiamycin-M_1 from DCCC.

temperature. The band of R_F 0.33 noted under UV light at 254 nm was scraped from the plate, and then dissolved in chloroform–methanol (9:1, v/v) and filtered to separate the sample substances from silica gel. The filtrate was evaporated to dryness at 40°C, and the residue was recrystallized twice from acetone. A 9.8-mg amount of pure M_1 as amorphous white crystals was obtained.

Determination of the partition coefficient and selection of the solvent system

To obtain the optimum partition coefficient (upper layer/lower layer), an 100-μg amount of crude VGM was added to 1 ml of the two-phase solvent mixture, and the mixture was shaken for 1 min. After separation, each layer (upper and lower) was pipetted out and subsequently subjected to HPLC with UV and fluorescence detection. M_1 was monitored by an UV detector, while VGM-S_1 (S_1), a main component of factor S, was monitored by the fluorescence detector. The partition coefficients of M_1 and S_1 were evaluated by measuring relative peak heights.

Microbiological assay

The antibacterial activity of VGM and M_1 was determined by a paper disk method with *Micrococcus luteus* ATCC 9341 having high sensitivity against VGM. The assay procedure was carried out according to the method of Katz *et al.*[5]. Each standard stock solution was prepared by dissolving VGM or M_1 in methanol to a concentration of 1000 μg/ml. A series of working standards of VGM or M_1 were prepared by diluting the stock solution in 50% methanol. Each filter-paper disk (10 mm in diameter) was impregnated by the aliquots (0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 μg/ml of VGM or M_1). The disks were placed on the surface of an agar plate. After incubating for 16-18 h at 37°C, the diameter of the inhibition zone formed around the disk was measured with a vernier caliper.

RESULTS AND DISCUSSION

Selection of solvent system for DCCC and separation of M_1

The various solvent systems suitable for DCCC have been listed by Ogihara *et al.*[2]. Hostettmann[3] has also reported that TLC is a suitable method to find an appropriate solvent system. In this study, HPLC was utilized to determine the partition coefficient of M_1 in different solvent systems with high accuracy and rapidity.

Based on an examination using almost the same solvent systems, benzene–chloroform–methanol–water (26:14:24:6) was the most suitable solvent system for droplet formation with partition coefficients of 0.8 and 0.2 for M_1 and S_1, respectively.

The use of the upper layer as a mobile phase in the ascending mode of DCCC was expected for earlier elution of M_1. As shown in Fig. 1, the elution of M_1 in DCCC gave a chromatogram with good separation under the conditions described above. On the other hand, another component in factor M, M_2, was found in the 400 to 450 ml fraction of the eluate. Furthermore, these fractions were monitored by HPLC. Consequently, factor S components were found not to be present in the fraction of M_1.

On the other hand, although a slight amount of pigment derived from the crude VGM was found in the fraction of M_1, it was separated by employing TLC and recrystallization as described.

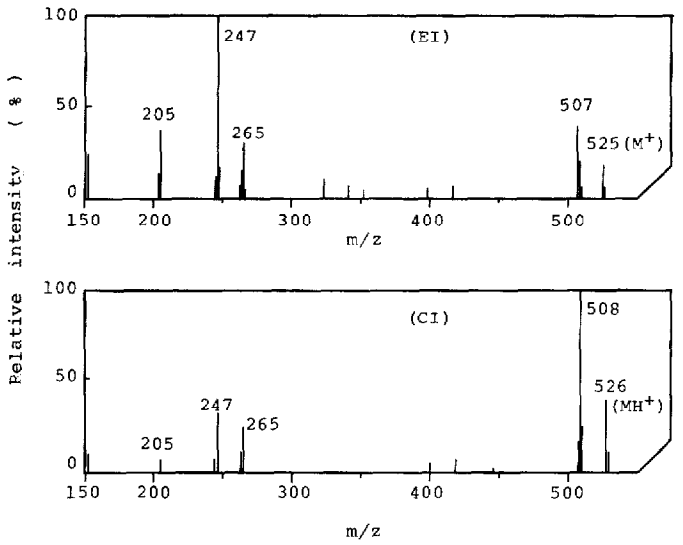

Fig. 2. Mass spectra of virginiamycin-M_1 measured by the EI and CI methods.

Confirmation of M_1

Fig. 2 shows the EI (A) and the CI mass spectrum (B) of the purified M_1. The parent peak at *m/z* 525 corresponds to the molecular weight of M_1 [M^+], while in the CI mass spectrum the peak at *m/z* 526 corresponds to the molecular weight of protonated M_1 [$M+H^+$]. The shift of the peak at *m/z* 507 in the EI mode is due to the loss of H_2O molecular ion from the parent peak, as is that of the peak at *m/z* 508 [$M+H-18^+$] in the CI mode. The other prominent fragmentation ions at *m/z* 265, 247 and 205, in Fig. 2 are typical ions of M_1. The molecular weight of M_1 separated by open-column chromatography as reported by Vanderhaeghe *et al.*[4] was 555. This discrepancy may be due to insufficient purification of M_1 by column chromatography.

The IR spectral pattern of M_1 was in good agreement with that reported by Vanderhaeghe *et al.*[4].

Correlation of the antibacterial activity between M_1 *and VGM*

Boon and Dewart[6] reported that the antibacterial activity of VGM depends on the synergism of factors M and S. The correlation between VGM and M_1 is therefore of interest. As shown in Fig. 3, the correlation between the inhibition zone of VGM

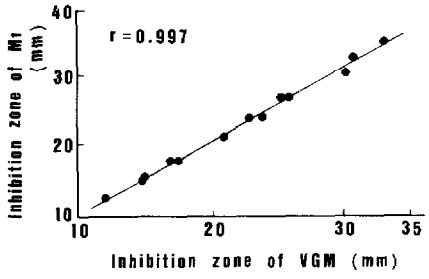

Fig. 3. Correlation between the antibacterial activity of VGM and M_1.

and that of M_1 exhibited a linear relationship with a correlation coefficient, r, of 0.993 ($n = 13$). Thus, from these results it is suggested that the amount of VGM in various samples can be determined by monitoring M_1 using HPLC.

CONCLUSION

Based on a study of the antibacterial activity of VGM and the main component, M_1, a good correlation, was found such that VGM could be determined by measuring M_1. DCCC has been used to obtain pure M_1 from crude VGM. From 50 mg of VGM, 9.8 mg of M_1 were obtained by using a two-phase system in DCCC.

ACKNOWLEDGEMENTS

We thank Nihon Zenyaku Kogyo for supplying the virginiamycin standard, and Dr. A. Tanaka for his help in the preparation of this paper.

REFERENCES

1 K. Okamoto, H. Yonezawa and N. Izumiya, *J. Chromatogr.*, 92 (1974) 147.
2 Y. Ogihara, O. Inoue, H. Otsuka, K. I. Kawai, T. Tanimura and S. Shibata, *J. Chromatogr.*, 128 (1976) 218.
3 K. Hostettmann, *J. Med. Plant Res.*, 39 (1980) 1.
4 H. Vanderhaeghe, P. Dijck, G. Parmentier and P. Somer, *Antibiot. Chemother.*, 7 (1957) 606.
5 S. Katz, J. Katz, J. Miller, R. Wang and R. Shapiro, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 569.
6 B. Boon and R. Dewart, *Analyst (London)*, 99 (1974) 19.