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CHROMATOGRAPHIC ISOLATION AND CHARACTERIZATION OF STREPTOGRAMIN ANTIBIOTICS

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SUMMARY

High-performance and medium-pressure liquid chromatographic techniques were developed for the rapid isolation, identification and characterization of the components from crude virginiamycin and viridogrisein. Two new minor components, denoted virginiamycin S_5 and viridogrisein II, were isolated. Their structures were determined by amino acid analysis and mass spectrometry. (I. Oberbäumer *et al.*, *Helv. Chim. Acta*, 65 (1982) 2280).

INTRODUCTION

Virginiamycin* and viridogrisein* are members of the streptogramin family of antibiotics. Virginiamycin is a mixture of several active components that fall into two groups, A and B, according to their structure and biological activity[1–3]. The crude mixtures are generally used as food additives. Viridogrisein contains group B components only. All members of group A are macrocyclic lactones of molecular weight about 500. Virginiamycin M_1 contains a 2,3-dehydroproline residue, which is substituted by D-proline in virginiamycin M_2. The structures of the streptogramins of group B (molecular weight in the range 800–900) are characterized by a macrocyclic peptide–lactone ring. The peptide–lactone ring of the virginiamycins contains six (Fig. 1a) amino acid residues, and that of viridogrisein seven (Fig. 1b); the N-terminal threonine is invariably N-acylated with 3-hydroxypicolinic acid. So far, two A components and four different B components have been isolated from virginiamycin[1–7]. Apart from some recent reports concerning congeners[8–10], viridogrisein[11–13] was generally considered to consist of a single B component.

The compounds of both groups are active mainly against Gram-positive bacteria; mixtures of the two groups show marked synergism[1–3]. The streptogramins act

* The following nomenclature and abbreviations are used: virginiamycin with no subscript represents the crude antibiotic mixture. The material used in this study consisted of the A components virginiamycin M_1 (VM_1) and M_2 (VM_2) and the B components virginiamycin S_1 (VS_1), S_4 (VS_4) and S_5 (VS_5). Viridogrisein (VE) consisted of the B components viridogrisein I (VE I) and II (VE II).

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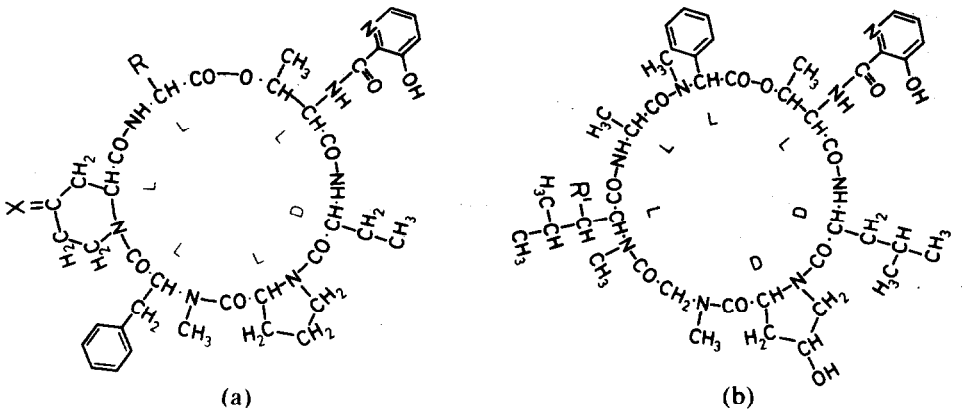

Fig. 1. (a) Structures of virginiamycin S_1 (R = C_6H_5; X = O) and virginiamycin S_5 (R = CH_3; X = OH, H); (b) Structures of viridogrisein I (R′ = CH_3) and viridogrisein II (R′ = H).

by binding to the bacterial ribosome and blocking protein biosynthesis. The B components, in addition, facilitate the transport of cations across phospholipid membranes[14]. The pure A and B components are not commercially available. As it is essential that all biological and biochemical investigations should be carried out with the pure A and B components, and only with mixtures prepared from these pure components, methods have been developed to allow their fast isolation and characterization by high-performance liquid chromatography (HPLC) and medium-pressure liquid chromatography (MPLC).

EXPERIMENTAL

Medium-pressure liquid chromatography

The apparatus consisted of a pump (Model 302; Gilson Medical Electronics, Middleton, WI, U.S.A.), a manometer module (Model 804; Gilson), an injector loop of capacity 0.1–1 ml (Abimed, Düsseldorf, F.R.G.) and a 120-ml glass column (300 × 23 mm I.D.), connected to a Uvicord S photometer equipped with a standard cuvette (optical path length 3 mm), a 276-nm filter and a recorder (LKB, Bromma, Sweden). For preparative separations a fraction collector (Model 2070 Ultrorac; LKB) was employed. LiChroprep 60 (25–40 μm for virginiamycin and 15–25 μm for viridogrisein) from Merck (Darmstadt, F.R.G.) was employed as the stationary phase. The filling of the column was performed according to the slurry method of Helmchen[15] and Flockerzi[16]. The antibiotics were dissolved in 1 ml of the mobile phase. Acetic acid (99–100%), chloroform and methanol were obtained from Merck.

High-performance liquid chromatography

The equipment consisted of the following: an SP 8000 A chromatograph, equipped with an SP 8300 fixed-wavelength UV detector (254 nm), optical path length 10 mm; an FS 970 fluorescence detector ($\lambda_{exc.}$ 295 nm and emission cut-off filter >389 nm) and a Valco injector with a 10-μl sample loop (Spectra Physics, Santa Clara, CA, U.S.A.). The ternary solvent feature of this chromatograph was utilized

to deliver the mobile phase. The oven temperature was 45°C and a flow-rate of 1 ml/min was used.

Analytical separations were performed on a Hyperchrome column (250 × 4.6 mm I.D.), filled with Shandon ODS Hypersil (5 μm) material (K. Bischoff, Stuttgart, F.R.G.). Phosphoric acid solution (pH 2.12, about 1 m*M*) was prepared from orthophosphoric acid (Merck) in doubly distilled water and filtered through a 0.45-μm Millipore filter. All the solvents were thoroughly degassed by purging with helium before and during the chromatographic runs. Methanol was of Uvasol grade and acetonitrile of LiChrosolv quality (Merck). The antibiotics were dissolved in methanol. After every run the column was flushed with methanol for 10 min and then re-equilibrated with the mobile phase.

Antibiotics

Virginiamycin was provided by Smith Kline (Brussels, Belgium) and viridogrisein by Parke, Davies (Detroit, MI, U.S.A.) and Bristol Labs. (Syracuse, NY, U.S.A.).

RESULTS AND DISCUSSION

HPLC and MPLC analyses were carried out on virginiamycin and viridogrisein to characterize both the crude antibiotics and the partially purified components. Selective detection of the B components was made possible by employing a fluorescence detector; an absorption detector exhibited similar sensitivity to A and B components.

With virginiamycin, no suitable separations by MPLC could be achieved with chloroform–methanol as the mobile phase. However, good results were obtained with chloroform–methanol–acetic acid (Fig. 2). It was found that the methanol content influenced the quality of the separations and the retention times of the components in a very sensitive way. Prior to preparative separations, it is advisable to perform test separations on the same column with small amounts of antibiotic in order to optimize the composition of the mobile phase.

Fig. 2a shows a preparative separation of virginiamycin by MPLC. Good results were obtained with maximum amounts of virginiamycin of up to 1 g per column. Although the retention times of the components were sensitive to the degree of loading for preparative runs, no effect on the elution order could be found. If it is required to isolate single components of high purity from preparative MPLC runs, re-chromatography of the initial fractions is advisable. The separation shown in Fig. 2a allowed the isolation of the virginiamycin components VS_1, VS_4, VS_5 and VM_1 with retention times of 12.1, 13.2, 17.2 and 28.5 min, respectively. The components were assigned on the basis of separate runs with pure individual components (Fig. 2b). Here the retention times of VS_1, VS_4 and VS_5 were 12.5, 13.9 and 19.6 min, respectively. In addition, the thin-layer chromatograms, mass spectra and ^1H NMR spectra of the isolated components were identical with those of the corresponding that were isolated employing procedures published elsewhere[4–6,17].

Virginiamycin S_5 is a new component that was not known until recently. Its structure was elucidated by amino acid analysis and mass spectrometry[18]. With reference to the main component VS_1, virginiamycin S_5 contains L-alanine instead of L-phenylglycine and L-*allo*-4-hydroxypipecolic acid instead of L-4-oxopipecolic acid

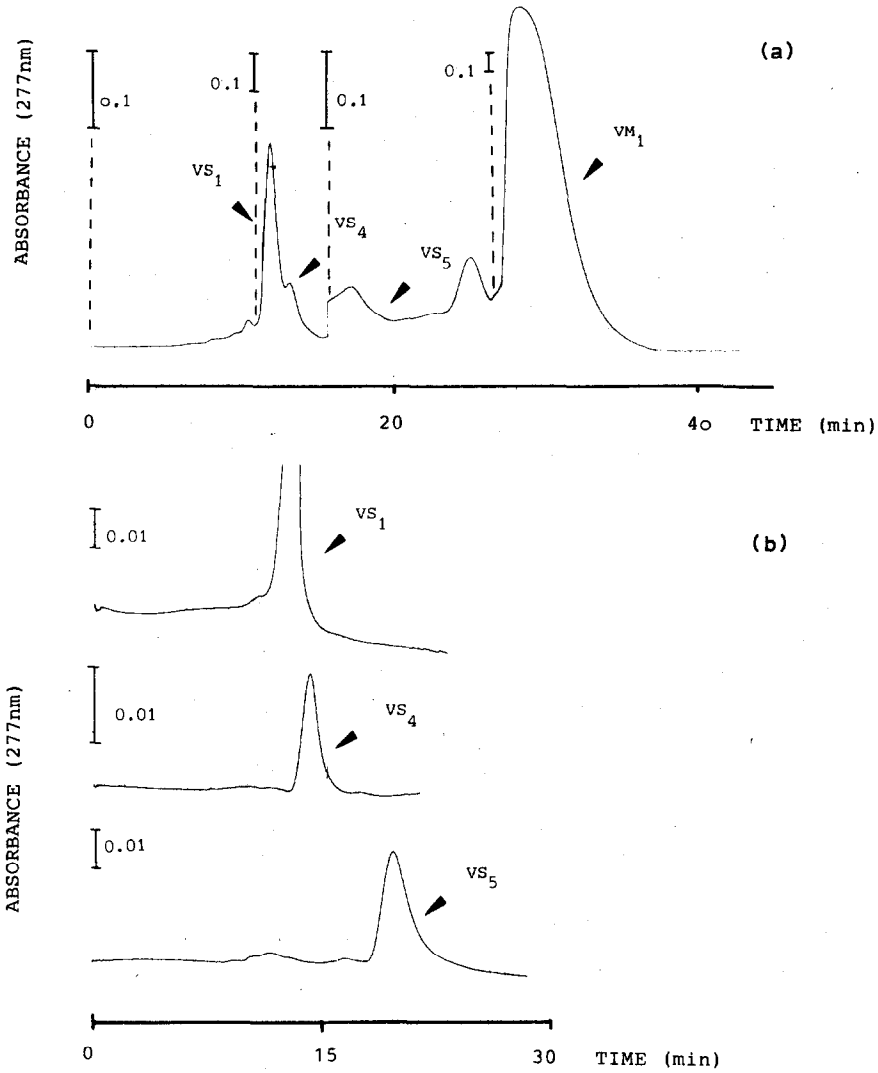

Fig. 2. Separation of (a) 64 mg of virginiamycin and (b) 1.81 mg of virginiamycin S_1, 0.62 mg of virginiamycin S_4 and 1.08 mg of virginiamycin S_5 by MPLC (absorption detection) on LiChroprep 60 with chloroform–methanol–acetic acid (100:2:12) as the mobile phase at 25°C.

(Fig. 1a). For analytical studies with the MPLC technique, the limit of detection under the present conditions was found to be about 100 μg for A and B components employing absorption detection and 10 μg for the B components when fluorescence detection was used.

Fig. 3 shows an analytical separation of crude virginiamycin by reversed-phase HPLC employing absorption and fluorescence detection. The best results were obtained with acetonitrile–methanol–phosphoric acid (pH 2.12) as the mobile phase. Whereas the absorption detector allowed the direct quantitative determination of the

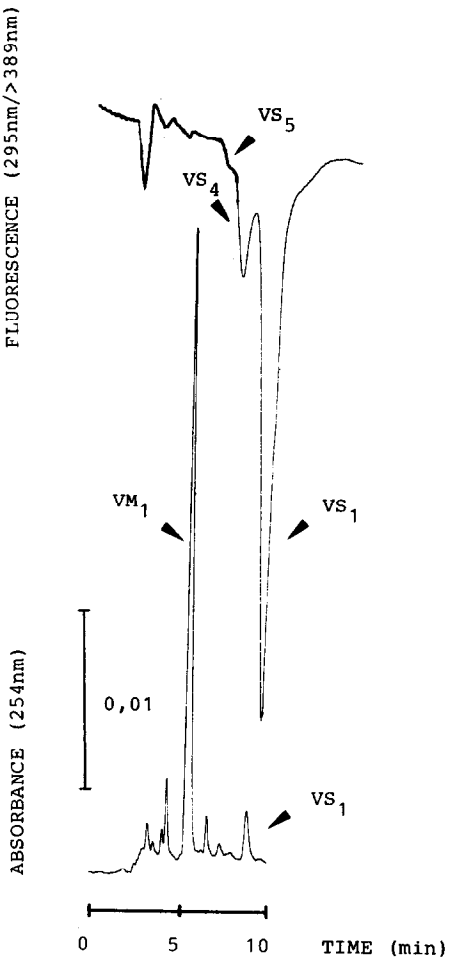

Fig. 3. Typical separation of 1 μg of virginiamycin by reversed-phase HPLC (absorption and fluorescence detection) with acetonitrile–methanol–phosphoric acid (pH 2.12) (44:10:46) as the mobile phase.

components VM_1 and VS_1 (retention times 5.5 and 9.7 min, respectively), fluorescence detection was required for the corresponding determinations of the B components VS_1, VS_4 and VS_5 (retention times 9.9, 8.4 and 7.6 min, respectively). The separation obtained with RP-8 columns were less satisfactory. Experiments were also performed with individual components. The retention times for VM_1, VS_1, VS_4 and VS_5 were 5.8, 9.8, 8.5 and 7.6 min, respectively. A linear relationship between detector intensity and sample concentration was observed between 0.2 and 100 μg/ml for VM_1 and between 1 and 100 μg/ml for VS_1. The detection limits correspond to these lower values for the absorption detector. Employing the fluorescence detector, the limits are at least ten times lower.

The samples of viridogrisein used in this study contained, in addition to the main component VE I, a minor component, VE II, which has recently been isolated by repeated column chromatography[17,18], a time-consuming procedure. MPLC sep-

arations of viridogrisein in the solvent system chloroform–methanol–acetic acid showed three different peaks (Fig. 4). The main peak with a retention time of 15.0 min is attributed to VE I, which exhibited a retention time of 15.4 min in a separate experiment (Fig. 4). The second peak (retention time 20.0 min) is due to VE II, which gave a retention time of 20.6 min in the separate run (Fig. 4). Quantitative analysis yielded a ratio of VE I to VE II of about 7.7, which is consistent with previous results[18]. It is evident that MPLC can also be used for the rapid isolation of VE II or for the enrichment of VE I. Compared with the main component VE I, viridogrisein II contains L-N-methylleucine instead of L-N,β-dimethylleucine, which was determined by amino acid analysis and mass spectrometry[18].

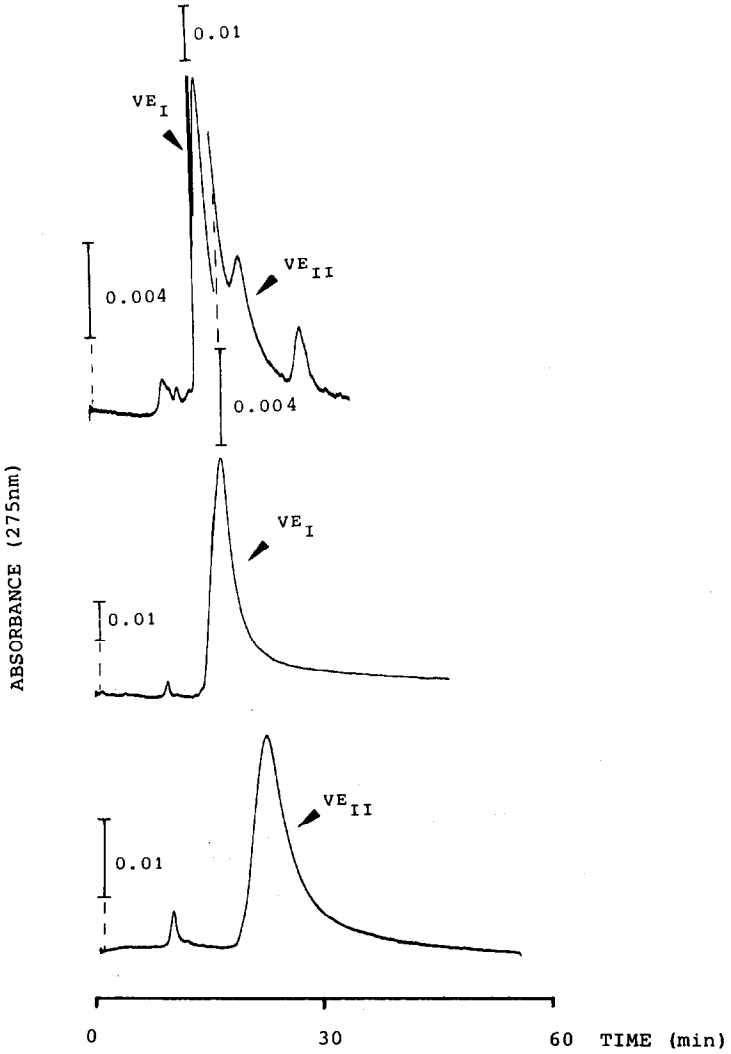

Fig. 4. Separation of 4 mg of viridogrisein, 3 mg of viridogrisein I and 3 mg of viridogrisein II by MPLC (absorption detection) on LiChroprep 60 with chloroform–methanol–acetic acid (100:1.5:12) as the mobile phase at 25°C.

CONCLUSION

These studies indicate that MPLC is a powerful technique for the rapid analytical characterization of streptogramin antibiotics. In addition, a very rapid and efficient preparative isolation of individual A and B components is possible by employing MPLC. The degree of purity of the components depends on the amount of crude mixture loaded, but can easily be improved by re-chromatographing selected fractions. Several of the components are unstable in the presence of polar solvents and silica gel, but there is relatively little decomposition with this method of isolation because a complete separation takes only about 30 min.

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