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DISPLACEMENT CHROMATOGRAPHY OF OLIGOMYCINS

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SUMMARY

Oligomycins A, B and C were separated by reversed-phase chromatography in the displacement mode on octadecylsilica columns. The carrier was a mixture of methanol and water and a saturated solution of palmitic acid in the carrier served as the displacer. The production rate was investigated as a function of the chromatographic conditions, *i.e.*, methanol concentration in the carrier, displacer concentration, flow-rate and the amount and concentration of the oligomycin mixture in the feed solution. A practical guide for method development is given.

INTRODUCTION

The introduction of new biotechnological methods for obtaining valuable biological products such as enzymes and antibiotics, has generated a need for new isolation techniques by means of which the valuable products can be separated from a complex mixture in high purity. High-performance liquid chromatography (HPLC) in the displacement mode, which was pioneered by Horváth and co-workers[1–3], has great potential for the economical purification and isolation of such products.

Displacement chromatography using HPLC columns has several advantages over elution chromatography: it allows the separation of hundreds of times greater amounts; owing to the relatively low flow-rate, only 5–10% of the amount of HPLC-grade solvent is needed; and the pure components are obtained in highly concentrated solutions, so that the recovery of the product is greatly facilitated. On the other hand, displacement chromatography has certain disadvantages: the overall time of the separation process is increased by a regeneration step, required for the removal of the displacer from the stationary phase; the theoretical basis of displacement chromatography is more complex[4]; and there are only a few examples of its application[5–10]. The paucity of these experiments has hampered method development

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and thus the widespread application of displacement chromatography to the large-scale separation and purification of biological products.

In this study, displacement chromatography was applied to the separation of oligomycins A, B and C, which are antibiotics produced by *Streptomyces diastatochromogenes*. Oligomycin A acts against the human pathogenic fungus *Blastomyces dermatitidis* and oligomycin B is a potent inhibitor of oxidative phosphorylation. Other properties of oligomycins have been reviewed by Show[11].

EXPERIMENTAL

Materials

Displacement development was carried out on LiChrosorb RP-18, 5 μm (Merck, Darmstadt, F.R.G.), packed in a 250 × 4.6 mm I.D. stainless-steel column. The Dimesil C-18 (10 μm) stationary phase on which the HPLC analyses of the fractions in the linear elution mode were carried out was packed by Chromatronix (Berkeley, CA, U.S.A.) into a 250 × 4.6 mm I.D. stainless-steel column.

The oligomycin mixture was obtained from Reanal (Budapest, Hungary). A 1-g amount of the crude oligomycin mixture was dissolved in 20 ml of methanol, than the components were precipitated by adding 80 ml of distilled water to the solution. The precipitate was filtered with a sintered porous-glass filter and then dried in a stream of nitrogen. Pure oligomycins A, B and C were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol (LiChrosolv) was supplied by Merck. Water was distilled twice from a glass still. Analytical-reagent grade palmitic acid was obtained from Reanal. The carrier was 75 and 80% (v/v) methanol in water. The displacer solution was prepared by dissolving the palmitic acid first in neat methanol and then adding the appropriate amount of water slowly in order to prevent precipitation of the palmitic acid. The concentration of the palmitic acid in the carrier solution ranged from 3 to 5 g/l. Conditions employed in the displacement chromatography of the oligomycins are summarized in Table I.

Apparatus

Displacement development was carried out with commonly used HPLC equipment, consisting of a Liquopump Model 312 (LaborMIM, Budapest, Hungary) with check valves from Altex (Berkeley, CA, U.S.A.). The sample was introduced by using a Model 7010 injection valve (Rheodyne, Berkely, CA, U.S.A.) with laboratory-made

TABLE I

CONDITIONS EMPLOYED IN THE DISPLACEMENT CHROMATOGRAPHY OF OLIGOMYCINS

Carrier		*Feed mixture*		*Displacer palmitic acid (g/l)*	*Flow-rate (ml/min)*
Methanol (%, v/v)	*Water (%, v/v)*	*Oligomycins (mg)*	*Carrier (ml)*		
80	20	15	2	5	0.30
75	25	25	5	3	0.10
75	25	50	5	3	0.10

2.00- and 5.00-ml sample loops. The effluent was monitored with a Millipore differential refractometer (Waters Assoc., Milford, MA, U.S.A.). Fractions were collected in a Pye eluent collector (Pye Unicam, Cambridge, U.K.). A Model 116 variable-wavelength UV detector (Gilson Medical Electronics, Villiers-le-Bel, France) was employed, and chromatograms were recorded with a Model OH-814/1 strip-chart recorder (Radelkis, Budapest, Hungary).

Separation procedure

The octadecylsilica column was equilibrated with the carrier at flow-rates of 1.00 and 0.10 ml/min for 20 and 10 min, respectively. Thereafter, the displacer solution was pumped through the injection valve. Pumping was stopped and the sample loop was filled with the oligomycin solution. The process was started by pumping the displacer solution through the sample loop into the column.

The separation was carried out under different conditions, as listed in Table I.

The effluent fractions were collected at 5-min intervals, *i.e.*, at flow-rates of 0.3 and 0.1 ml/min fractions of 1.5 and 0.5 ml, respectively, were obtained. Of each fraction, 10-μl aliquots were diluted with 100 μl of mobile phase and 20-μl samples were analysed.

Column regeneration

The column was regenerated with methanol–water with 5 m*M* K_2HPO_4 buffer (pH 8.5) (9:1) at a flow-rate of 1.00 ml/min for 20 min. Thereafter, the column was washed first with water and then with methanol.

Analysis of the fractions

The oligomycins were analysed by elution chromatography on the Dimesil C-18 (10 μm) (250 × 4.6 mm I.D.) column using 85% aqueous methanol as the mobile phase, degassed ultrasonically under vacuum, at a flow-rate of 1 ml/min. The column effluent was monitored at 225 nm.

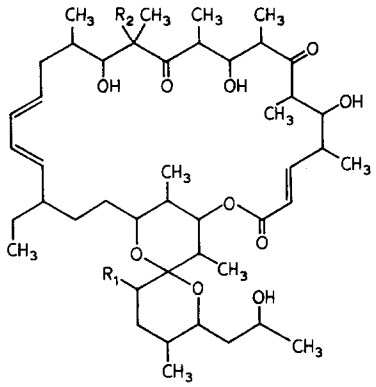
RESULTS AND DISCUSSION

The chemical structure of the oligomycins[1,2] is shown in Fig. 1. These antibiotics exhibit a highly hydrophobic character. The solubility of oligomycin A in water is only 0.02 g/l. Therefore, a mobile phase rich in organic solvent was selected in order to dissolve appreciable amounts of oligomycins and elute them from an octadecylsilica stationary phase. A typical chromatogram of the oligomycin mixture used in this study is shown in Fig. 2.

The development of the method for the separation of oligomycins by displacement chromatography entailed the following steps:

(i) selection of a displacer substance that has a significantly longer retention time than the compounds to be separated and fulfills the requirements discussed in detail by Horváth *et al.*[2];

(ii) selection of the carrier that does not elute components with a retention factor smaller than that of the displacer and that preferably contains volatile solvent components only;



Oligomycin A $C_{45}H_{74}O_{11}$ R_1=H R_2=OH
B $C_{45}H_{72}O_{12}$ R_1=OH R_2=OH
C $C_{45}H_{74}O_{10}$ R_1=H R_2=H

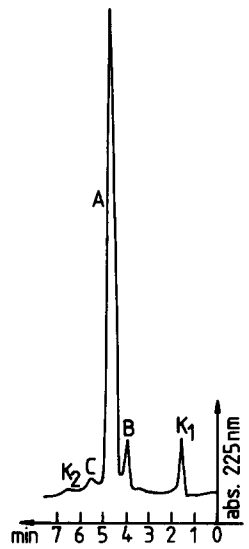


Fig. 1. Structure of oligomycins. Oligomycin A, R_1 = H, R_2 = OH; B, R_1 = OH, R_2 = OH; C, R_1 = H, R_2 = H.

Fig. 2. Separation of oligomycins A, B and C. Column, Dimesil C-18 (250 × 4.6 mm I.D.); mobile phase, 85% aqueous methanol; flow-rate, 1.00 ml/min; detector setting, 225 nm. K_1 and K_2 are unknown impurities.

(iii) optimization of the production rate, which depends on the organic solvent concentration of the carrier, the flow-rate, the amount and concentration of the feed mixture and the displacer concentration.

For example, the number of fractions containing more than one product can be decreased by using a very low flow-rate and a low organic solvent concentration of the carrier. However, this increases the time required for the separation accordingly.

Selection of the displacer

In selecting the displacer we applied the following considerations: (i) the displacer should have a more hydrophobic character and be bound to the stationary phase more strongly than any components of the mixture to be separated; and (ii) the displacer solution in the carrier should be of sufficiently high concentration to obtain a relatively high velocity of the displacement front and rapid development of the displacement trains.

Study of the retention behaviour of potential displacers by linear or gradient elution chromatography provides valuable information for the selection. A substance having a longer retention time than those of the components to be displaced in a given chromatographic system can be expected to be an adequate displacer when the pertinent adsorption isotherms are not known.

The retention behaviour of a few potential displacers, such as cetyltrimethylammonium bromide, α-naphthol, benzylbenzoate, hexachlorophene, *n*-octanol and benzaldehyde, as potential displacers was investigated by using the octacecylsilica

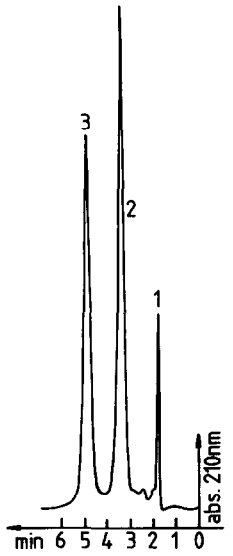


Fig. 3. Retention of palmitic acid and oligomycins. Column, Dimesil C-18 (250 × 4.6 mm I.D.); mobile phase, 100% methanol; flow-rate, 1.00 ml/min; detector setting, 210 nm. 1 = Impurity of palmitic acid; 2 = unresolved oligomycin A, B and C; 3 = palmitic acid.

column with aqueous organic eluents. All of these substances, however, were eluted at retention times shorter than those of the components of the oligomycin mixture.

We have found that of the substances investigated only palmitic acid was more strongly retained than the oligomycins, as shown in the chromatogram in Fig. 3. On the other hand, with 85% methanol as the aqueous mobile phase, the palmitic acid could not be eluted. These observations suggested that palmitic acid was a promising displacer for the separation of the mixture under consideration.

An advantage of palmitic acid as the displacer is the presence of the carboxyl group in the molecule, which can be ionized in alkaline solution, rendering the molecule much more hydrophilic, so that its removal from the stationary phase in the regeneration step is facilitated.

Selection of the carrier

When the methanol concentration in the mobile phase was decreased from 100 to 80% the retention of oligomycins increased significantly in the elution mode, and the retention factors increased to values ranging from 25 to 55. This observation suggested that the carrier for the displacement development should contain about 80% or slightly less of methanol in order to minimize elution. A lower methanol concentration in the carrier was not considered because of the low water solubility of oligomycins and palmitic acid.

The use of 80% aqueous methanol as the carier allowed the preparation of a displacer solution of relatively high concentration (5 g/l), and the carrier was also a good solvent for the oligomycins. With this carrier, the time of the separation was relatively short, but the efficiency of the separation was low because of intermixing of the adjacent bands.

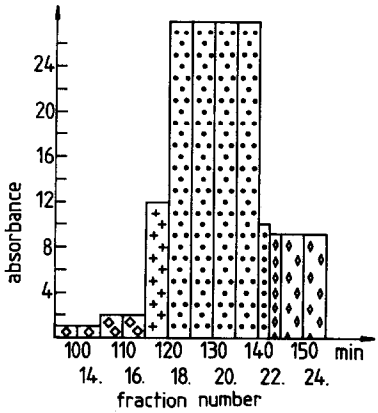


Fig. 4. Displacement chromatogram of oligomycins. Column, LiChrosorb RP-18, 5 μm (250 × 4.6 mm I.D.); carrier, 75% aqueous methanol; flow-rate, 0.10 ml/min; displacer, 3 g/l palmitic acid; feed mixture, 25 mg of oligomycins in 5 ml of carrier. Key: ○, oligomycin A; +, B; ◊, C; ◇, K_1 (unknown impurity).

By using 75% aqueous methanol as the carrier and a displacer concentration of 3 g/l, the separation time increased owing to the lower displacer concentration. However, the concentration of the separated components in the effluent decreased (Fig. 4). On lowering the methanol concentration of the carrier the number of fractions containing more than one feed component decreased significantly. These findings suggest that lower methanol concentrations in the carrier would give better separations. However, as mentioned above, the low solubility of the species did not make it practicable to use carriers having methanol concentrations lower than 75%.

The production rate has been defined by Horváth *et al.*[1] as the total amount of the pure compounds in the effluent divided by the sum of feed time and the breakthrough time of the displacer. It is an important measure of the economy of large-scale purification by chromatography. We attempted to increase the production rate by doubling the feed concentration. However, this resulted in a significant number of mixed fractions, indicating a low separation efficiency. It appears that the 25-cm column was not long enough for the development of the displacement train with such a high load under the operating conditions employed.

CONCLUSIONS

Displacement chromatography of oligomycins has the potential for the economical large-scale separation of a crude mixture of oligomycins A, B and C. It has been demonstrated that displacement development with a given HPLC column allows the separation of several hundred times greater amounts that is possible in the elution mode and requires only 5–10% of the solvent volume. The pure components can be obtained in highly concentrated solutions and hence the product recovery is facilitated. With the octadecylsilica column, optimal operating conditions were obtained by using 75% aqueous methanol as the carrier and 3 g/l palmitic acid solution in the carrier at a flow-rate of 0.1 ml/min. The advantage of using palmitic acid as the displacer is that it can be easily and rapidly removed from the stationary phase with a slightly alkaline regenerant.

ACKNOWLEDGEMENT

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