Journal of Chromatography, 268 (1983) 315–320
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,010

Note

Preparative reversed-phase high-performance liquid chromatography of monensin A and B sodium salts

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(Received May 26th, 1983)

The most important metabolites produced by *Streptomyces cinnamonensis* are polyether antibiotics represented primarily by monensin A and B sodium salts (monensin A and B) (Fig. 1)[1–4]. These compounds have previously been isolated by classical column chromatography (CC) and preparative thin-layer chromatography (TLC)[5]. CC seems to be the more suitable method for isolating of gram amounts of monensins but it requires fractionation of the eluent and subsequent analysis of monensins in particular fractions by TLC after detection with vanillin–sulphuric acid spray[3]. Moreover, the chromatographic run may be slow, taking tens of hours.

The aim of this work was to separate monensin A and B from the crystalline monensin complex on the analytical and preparative scale using reversed-phase high-performance chromatography (RP-HPLC) and UV detection. The developed method is fast (an average preparative chromatographic run takes 55 min) and monensins are detected directly in the effluent. Gram amounts of monensins for physico-chemical studies and for biosynthetic and biological experiments *in vitro* and *in vivo* can be prepared in this way.

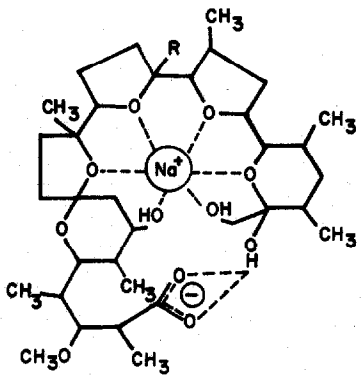

Fig. 1. Structural formula of monensin A (R = C_2H_5) and B (R = CH_3).

0021-9673/83/$03.00

EXPERIMENTAL

Sample

A crystalline monensin complex was prepared as described by Haney *et al.*[6]. The sample was dissolved in the mobile phase (methanol–water) prior to injection. Methanol–water mixtures were helium degassed.

Analytical mode

Analytical chromatography was carried out on an SP-8000 liquid chromatograph (Spectra-Physics, Santa Clara, U.S.A.) equipped with a 500-μl sample loop. The stainless-steel column (340 × 4 mm I.D.) was packed with Separon C18 (Laboratorní přístroje, Prague, Czechoslovakia), which is a 10-μm reversed-phase packing. The mobile phase (methanol–water) flow-rate was maintained at 1 ml · min^{-1}. An SP-770 variable-wavelength UV detector (Schoeffel Instruments, Westwood, NJ, U.S.A.) with an 8-μl flow-through cell was set at 215 nm and the absorbance range 0.02–0.4 was used. Chromatograms were recorded on a strip-chart recorder at a chart speed of 0.5 cm · min^{-1} and a setting of 10 mV.

Preparative chromatography

A Chromatospac Prep 100 preparative chromatograph (Jobin-Yvon, Longjumeau, France) with axial compression of the chromatographic bed (33.6 × 8 cm I.D.) was used for the isolation of monensins from their mixtures. The mean particle diameter of the Separon C18 reversed-phase packing was 15 μm (Laboratorní přístroje) and the mobile phase flow-rate was 80 ml · min^{-1}. A Holochrome H/MD variable-wavelength detector (Gilson, France) with a 40-μl flow-through cell was set at 215 nm and at an absorbance range of 0.1–1.0. The input sensitivity of the recorder was 10 mV full-scale and the chart speed was 0.5 cm · min^{-1}.

The samples were dissolved in the mobile phase (methanol–water, 88:12) with a constant volume of 25 ml. Depending on the detector response (see Fig. 5) three fractions were collected: monensin A, monensin B and their mixture. The hold-up volume of the column in both the analytical and preparative modes was determined by injecting water and evaluating the time elapsed between injection and elution. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

The degree of separation of monensin A from B is described by an empirical factor[7] $P = fg^{-1}$ (see Fig. 5 for the meaning of the variable P). The dependence of P on the mobile phase composition for 0.1 mg of a mixture of monensin A and B was evaluated on an analytical column. It is obvious from Fig. 2 that $P = 0.9$ (*i.e.*, P is decreased by 10% in comparison with the baseline separation) at 8% of water in methanol. Further, the dependence of P on the amount of solute injected was measured [the mobile phase composition was methanol–water (88:12)]. The value $P = 0.9$ (10% relative decrease) is achieved when 3 mg of the mixture injected (see Fig. 3). Fig. 4 illustrates an example of the analytical separation of monensin A and B using methanol–water (88:12) and injecting 0.1 mg of the mixture. The data obtained in an analytical column (Figs. 2 and 3) served as a guide for the selection of the experi-

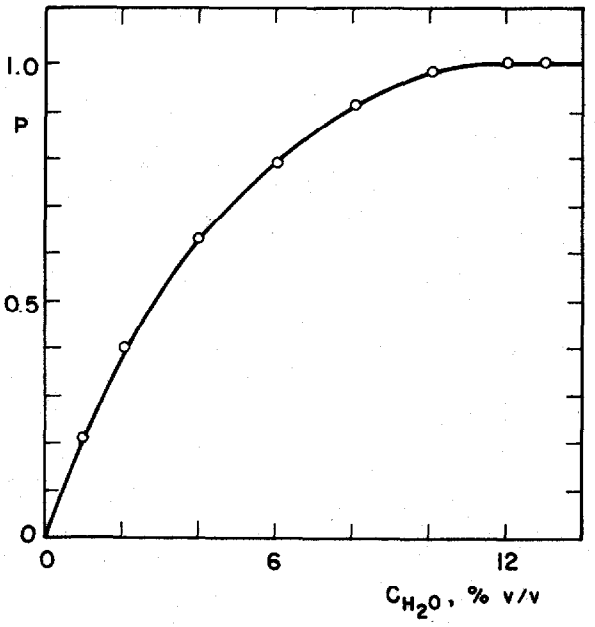

Fig. 2. Relationship between P and content of water in the mobile phase. Column, 340 × 4 mm I.D., packed with Separon C18 (10 μm); mobile phase, methanol–water; flow-rate, 1 ml · min^{-1}; detection, UV (215 nm).

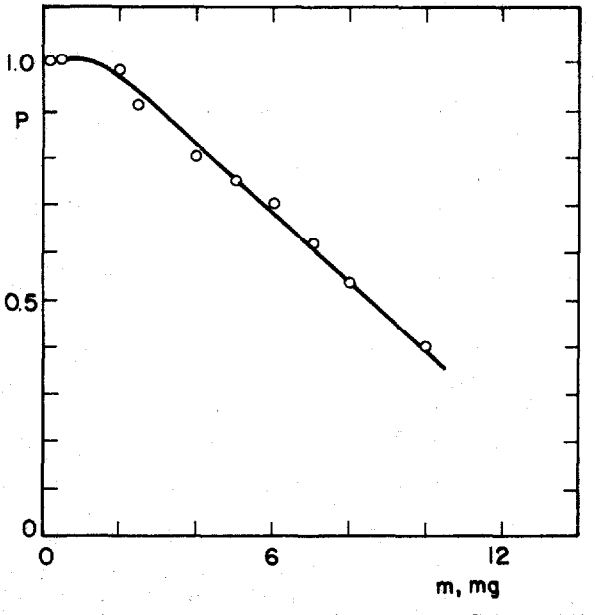

Fig. 3. Relationship between P and sample size. Column, 340 × 4 mm I.D., packed with Separon C18 (10 μm); mobile phase, methanol–water (88:12); flow-rate, 1 ml · min^{-1}; detection, UV (215 nm).

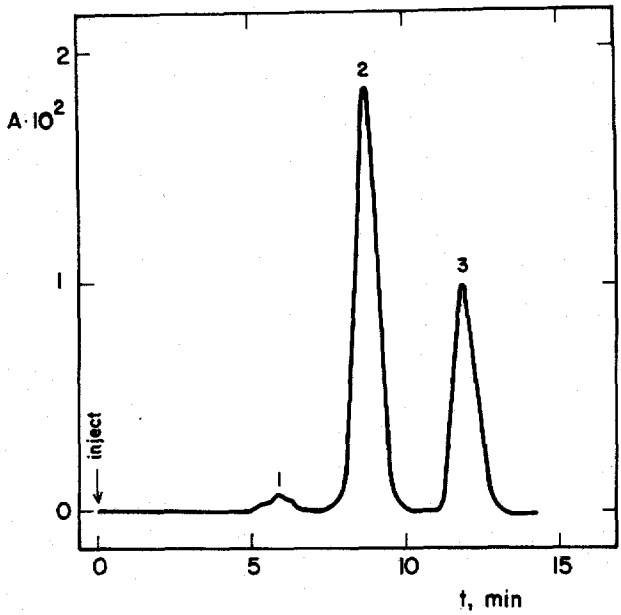

Fig. 4. Analytical separation of a mixture (0.1 mg) of monensin A and B. Column, 340 × 4 mm I.D., packed with Separon C18 (10 μm); mobile phase, methanol–water (88:12); flow-rate, 1 ml · min^{-1}; detection, UV (absorbance *A*) (215 nm). 1 = Unknown compounds; 2 = monensin B; 3 = monensin A.

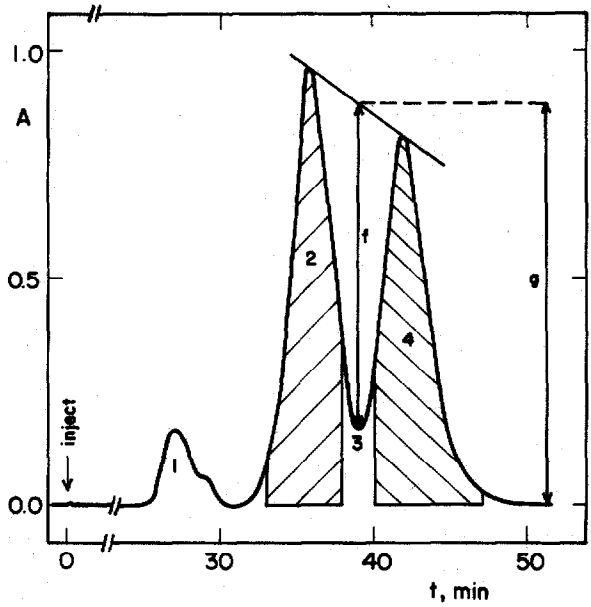

Fig. 5. Preparative separation of a mixture (1 g) of monensin A and B. Column, 33.6 × 8 cm I.D., packed with Separon C18 (15 μm); mobile phase, methanol–water (88:12); flow-rate, 80 ml · min^{-1}; detection, UV (absorbance *A*) (215 nm). 1 = Unknown compounds; 2 = monensin B; 3 = mixture of monensin A and B; 4 = monensin A; *f,g* = length of abscissae used for calculation of factor *P*.

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR ANALYTICAL AND PREPARATIVE COLUMN

V = volume of column; V_0 = hold-up volume; t_0 = hold-up time; F = flow-rate; t_c = time of chromatographic run; c_{H_2O} = concentration of water in methanol.

Column	V (cm^3)	V_0 (cm^3)	t_0 (*min*)	F ($ml \cdot min^{-1}$)	t_c (*min*)	c_{H_2O} (%, *v/v*)
Analytical	4.2	2.4	2.4	1.0	14.0	12.0
Preparative	1690.0	944.0	11.8	80.0	55.0	12.0

mental conditions for the preparative column. With a mobile phase containing 12% of water, the conditions were not only suitable for the desired separation but also permitted economical use of the preparative chromatograph. It was possible to make two runs using 11 l of the mobile phase (the volume of the reservoir) without excessive waste and saving the equilibration time for the second run. The ratio of the net volume of the preparative column to that of the analytical column (see Table I) is about 420. According to this value the maximum loading of the mixture for the preparative column was approximated to 1260 mg. Nevertheless, preparative trials were carried out for injected amounts of 50 mg (P = 1.0), 850 mg (P = 0.88), 1000 mg (P = 0.8) and 2000 mg (P = 0.6). Fig. 5 represents an example of the preparative separation of 1 g of the crystalline mixture of monensins.

Table I gives an overview of the chromatographic conditions for the analytical and preparative separations. The reversed-phase chromatographic technique was used throughout because of its superiority over the normal-phase method (*e.g.*, silica gel with benzene–ethyl acetate). There is no loss of sample on the column (under the experimental conditions described), the equilibration is not as time consuming as on silica gel columns and the solutes are easily separated from the mobile phase fractions by rotary evaporation of methanol under vacuum and subsequent precipitation in the water residue. Finally, the mobile phase used (methanol–water) was transparent down to 215 nm. With the samples of monensins that were exposed in the solution to temperatures higher than 40°C, shift changes were observed by nuclear magnetic resonance (NMR) spectroscopy for certain carbon atoms as a result of epimerization[8]. The mass spectra of these samples revealed a higher proportion of monensin free acid[9].

The yield of a single chromatographic run under the experimental conditions used was 0.5 g of monensin A, 0.4 g of monensin B and 0.07 g of a mixture of the two (the amount of monensin complex injected was 1 g). Minor components of the mixture were not identified.

The purity of the monensin products was determined by TLC on silica gel and by RP-HPLC. The authenticity of monensins was proved by ^{13}C NMR spectroscopy comparing the chemical shifts with literature data[10]. The similarity index[11] was 0.986 for monensin A and 0.997 for monensin B. The mass spectra of the isolated monensins were identical with those reported in the literature[9].

ACKNOWLEDGEMENTS

Thanks are due to Mr. P. Sedmera and Dr. J. Vokoun for measurement of the NMR and mass spectra and to Mr. J. Jiroušek for his technical assistance.

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