Journal of Chromatography, 604 (1992) 285–289
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 191

Short Communication

Optimization of high-performance liquid chromatographic conditions for the determination of cyclosporins A, B and C in fermentation samples

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(First received November 7th, 1991; revised manuscript received March 17th, 1992)

ABSTRACT

Variations in the retention behaviour of cyclosporins A, B and C, which have very similar structures, were studied with respect to mobile phase composition, temperature, stationary phase and UV detection wavelength. Cyclosporins A, B and C were well separated with a Supelco C_8 column (7.5 cm × 4.6 mm I.D.) at 60°C using acetonitrile–water (50:50) containing 0.01% of orthophosphoric acid at a flow-rate of 1 ml/min with UV detection at 202 nm. Cyclosporins A, B and C obtained from an indigenous isolate of *Tolypocladium* sp. were determined and it was found that the major component was cyclosporin A followed by C and B. The advantage of this method is that the interference of closely eluting peaks can be avoided as there is a good separation between the peaks and the analysis can be carried out economically as the solvent consumption is low.

INTRODUCTION

Cyclosporins represent a group of cyclic peptides composed of eleven amino acids. They are produced by fungi of the genus *Tolypocladium* in submerged culture [1]. Cyclosporins A, B and C differ in their structures only at the second amino acid. They are neutral and hydrophobic in nature [2]. Cyclosporin A is a promising immunosuppressive drug which is being used extensively in organ transplantation and the treatment of autoimmune diseases [3].

Various methods have been reported for the determination of cyclosporin A [4–9]. Owing to the close similarity of their structures, the determination of the cyclosporins A, B and C in fermentation samples is complicated. However, their resolution is of the utmost importance for the identification and determination of cyclosporin A. The optimization of the analytical conditions for a high-performance liquid chromatographic (HPLC) procedure for the separation of cyclosporin A, B and C obtained from an indigenous isolate of *Tolypocladium* sp. [10] is presented here.

EXPERIMENTAL

A Shimadzu (Tokyo, Japan) Model LC-3A high-performance liquid chromatograph equipped with a Model SPD-2A variable-wavelength UV detector, a Model CTO-6A column oven and a Model C-R3A data processor was used. All sample injections were

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made with a Rheodyne (Cotati, CA, USA) Model 7125 injector using a Hamilton (Reno, NV, USA) microsyringe.

Standard samples of cyclosporin A, B and C used for determining retention indices were kindly supplied by SPIC Pharmaceuticals (Madras, India). Methanol and acetonitrile were of HPLC grade (S.D. Fine-Chem, Boisar, India). Analytical-reagent grade orthophosphoric acid was obtained from Ranbaxy (Punjab, India). HPLC-grade water was obtained with a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

Standard samples were prepared by dissolving pure cyclosporins A, B and C in acetonitrile (0.3 mg/ml). The fermentation samples were prepared by extracting the mycelial biomass obtained after 21 days of static fermentation of the fungus *Tolypocladium* sp. [10] with methanol (1:2, w/v) and filtering through a 0.2-μm filter unit (Millex-FGS; Millipore).

The optimization of the analytical conditions was carried out with respect to mobile phase composition, pH, temperature, analytical column and UV detection wavelength.

Mobile phases were prepared with different proportions of acetonitrile and water and containing three different orthophosphoric acid concentrations (0.001, 0.01 and 0.1%), leading to variations in pH in the range 2.2–3.5. Experiments were conducted at three different temperatures (40, 60 and 70°C) and also using different columns differing in their packing materials and dimensions, *viz.*, Resolve C_{18}, 5 μm (15 cm × 3.9 mm I.D.) (Waters–Millipore, Milford, MA, USA) and Supelco C_8, 3 μm (7.5 cm × 4.6 mm I.D.) (Supelco, Gland, Switzerland). Analyses were carried out at different wavelengths from 202 to 214 nm at 0.01 a.u.f.s. The capacity factor, k' [$k' = (t_R - t_0)/t_0$, where t_R is the retention time of the solute and t_0 is the retention time of an unretained compound] [11] and the selectivity factor, α ($\alpha = k'_2/k'_1$), were calculated. All analyses were carried out at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

The effects of the mobile phase composition and the nature of the stationary phase on the retention of cyclosporins A, B and C at three column temperatures are shown in Figs. 1 and 2.

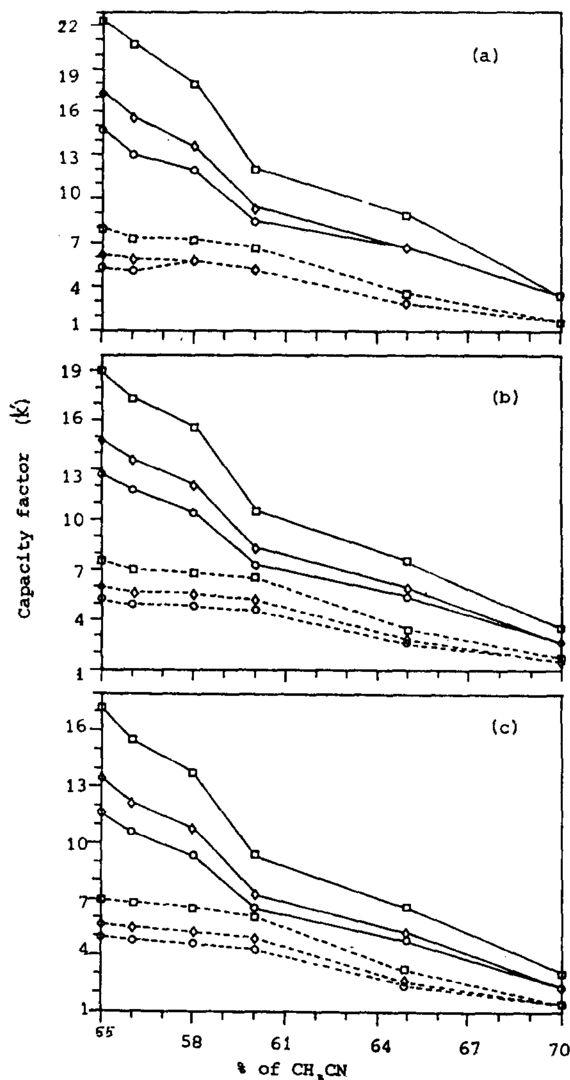

Fig. 1. Variation in k' for cyclosporins A, B and C at (a) 40°C, (b) 60°C and (c) 70°C with respect to acetonitrile concentration in the mobile phase containing 0.01% of H_3PO_4 and the type of column used. Solid lines, Resolve C_{18} column; dashed lines, Supelco C_8 column. Cyclosporins: □ = A; ◇ = B; ○ = C.

The variation of the capacity factor at three levels of orthophosphoric acid concentration and column temperature with respect to two columns (C_{18} and C_8) and different percentages of acetonitrile in water (70, 65, 60, 58, 56 and 55%) as mobile phase was studied. At a column temperature of 40°C with 70% acetonitrile, the cyclosporins were eluted together with α = 1. As the percentage of acetonitrile was

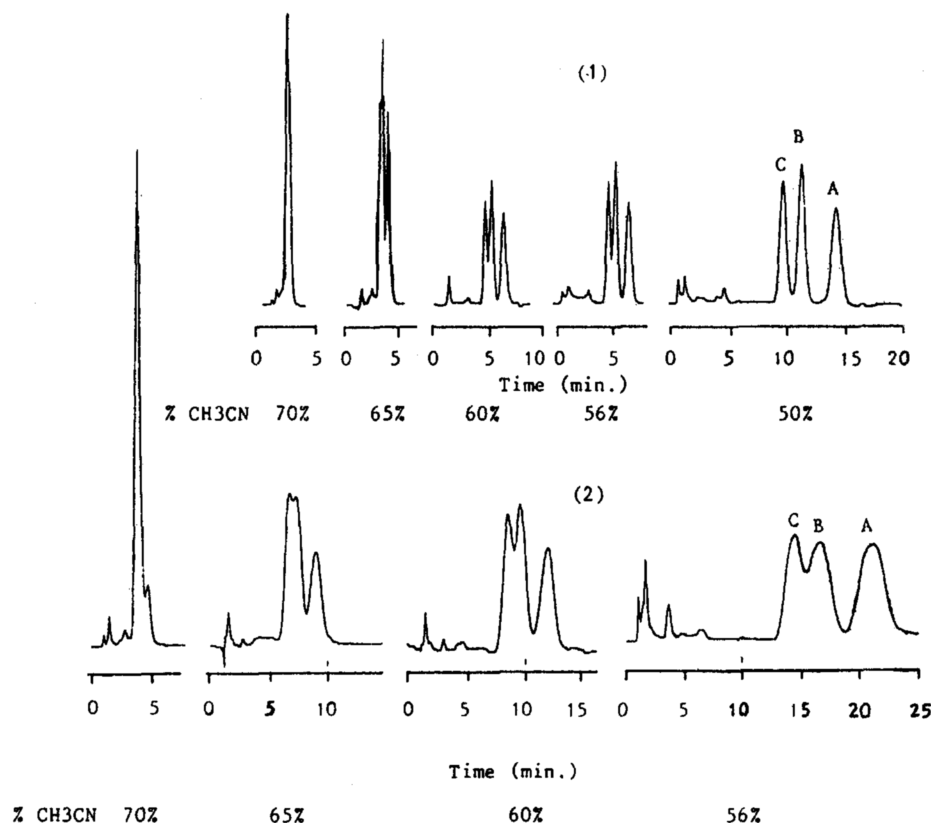

Fig. 2. HPLC profiles of a mixture of cyclosporins A, B and C (0.3 μg per injection) with various percentages acetonitrile in the mobile phase using (1) the Supelco C_8 column and (2) the Resolve C_{18} column at 60°C.

decreased the retention times of the cyclosporins became longer and the separation improved. The separation was very poor and peak broadening was observed at 40°C (Fig. 1) compared with the results obtained at the column temperatures of 60°C (Fig. 1b) and 70°C (Fig. 1c), which showed a reduction in retention time and better separation. However, the changes in selectivity were insignificant in most instances.

Even though the results of the analysis with the Resolve C_{18} column showed a good resolution, the higher values of the capacity factors indicate the need for a longer time of analysis or higher flow-rates with low percentages of acetonitrile. Compared with the Resolve C_{18} column, the Supelco C_8 column with a shorter length gave shorter retention times with very good peak separation and narrower peaks (Fig. 2). The analysis time is shorter with good separation and low solvent consumption. Subsequent experiments were therefore performed using only the Supelco C_8 column with 52% and 50% of acetonitrile in the mobile phase containing 0.01% of H_3PO_4 at 60°C in order to effect further improvements in the separation. With acetonitrile–water (50:50) at a flow-rate of 1 ml/min a very good separation was obtained for cyclosporins A, B and C using this column whereas a higher flow-rate has to be used with the C_{18} column to avoid longer retention times. With respect to the acid concentration, better results were observed with concentrations of 0.01% and 0.1% than 0.001%. The analyses at 60 and 70°C gave good results but considering the column lifetime, 60°C and 0.01% of orthophosphoric acid were selected as the optimum temperature and acid concentration for the determination of cyclosporins.

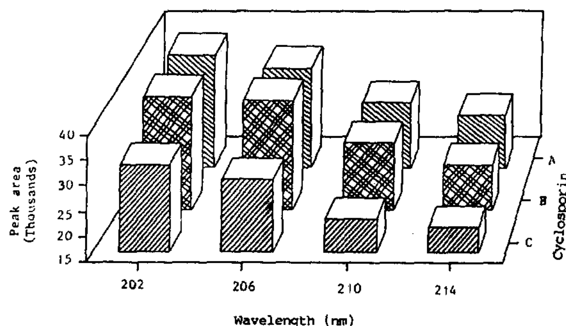

Fig. 3. Detection of cyclosporins A, B and C (0.15 μg per injection) at different UV wavelengths.

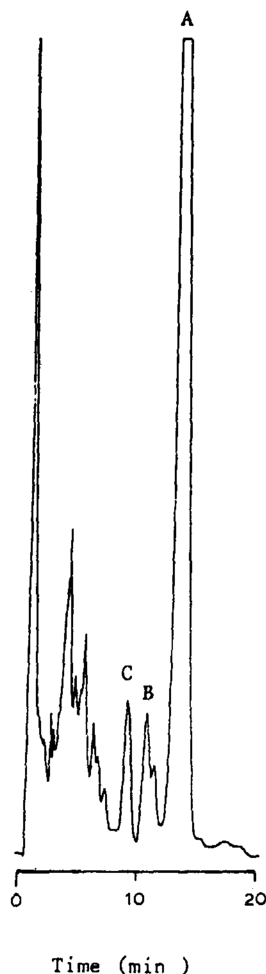

Fig. 4. HPLC profile of a crude fermentation sample showing the presence of cyclosporins A, B and C.

In order to establish the optimum detection wavelength, analyses were carried out at 202, 206, 210 and 214 nm and the corresponding peak areas were compared for the same sample amount (0.15 μg). Maximum peak areas were observed for cyclosporins A, B and C at 202 nm; the peak areas were reduced at higher wavelengths more for cyclosporin C than B and A (Fig. 3).

After optimizing the chromatographic conditions for the determination of cyclosporins A, B and C as acetonitrile–water (50:50) containing 0.01% of orthophosphoric acid as mobile phase and using a Supelco C_8 column at 60°C, fermentation samples were analysed. The chromatogram in Fig. 4 shows that the major component of the fermentation samples was cyclosporin A, followed by cyclosporin C and B.

The advantage of this method is that in quantitative analysis the interference of closely eluting peaks can be avoided as there is a good separation between the peaks. Analyses can be carried out economically as the solvent consumption is low.

ACKNOWLEDGEMENTS

The authors are highly indebted to Dr. V. Dhanda, Director, and Dr. P. K. Rajagopalan, former Director, VCRC, Pondicherry, for provinding the necessary facilities. They are grateful to Dr. M. Kalyanasundram, Assistant Director, for valuable suggestions. Technical assistance provided by Mr. D. Jayakumar and S. Venugopal is gratefully acknowledged.

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