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HIGH-SPEED LIQUID CHROMATOGRAPHY WITH CONVENTIONAL INSTRUMENTS FOR THE DETERMINATION OF CYCLOSPORIN A, B, C AND D IN FERMENTATION BROTH

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SUMMARY

For the separation and determination of the closely related, cyclic undecapeptides cyclosporin A, B, C and D in samples from fermentations, a high-speed liquid chromatographic method with conventional apparatus has been developed, based on a separation with a 3-μm Nucleosil C_8 column and acetonitrile–water–phosphoric acid (700:300:0.1) as eluent at 70°C. The analysis time of about 3.5 min, the use of a sampler for 195 vials and a laboratory data system offer a high degree of automation and analytical economy. The coefficient of variation is about 0.8–1.5% (n = 10). The rather low plate counts for the cyclosporins are due to the structures and high molecular weights of these substances.

INTRODUCTION

Conventional high-performance liquid chromatography (HPLC) is an often automated, but a rather slow separation method. The incentives to improve it are based on economic grounds (more analyses in less time) and competition from another fast separation method, which can be fully mechanized, namely high-performance thin-layer chromatography (HPTLC). Efforts to increase the analytical economy of HPLC by means of column switching-assisted methods, *e.g.*, box-car chromatography[1], have been carried out. On the other hand, according to theoretical considerations, a reduction in particle size of the sorbent should result in faster mass transfer, lower diffusion and higher plate counts, which means shorter columns and higher speed.

Since 1981 it has been shown that short columns, filled with 5-μm reversed-phase particles, in combination with UV detectors, equipped with low-volume flow cells and fast electronics, result in separation times as low as 1 min[2–4]. Furthermore, a UV detector with a 3-cm cartridge (5-μm particles) and an especially modified flow cell has been developed, enabling extremely fast separations[5].

All these high speed- and very high speed- or super speed-LC methods can be adapted to the analysis of substances with small to medium molecules and which are easily separated from each other due to their different structures. Therefore it is of interest to determine whether the separation time for the mixture of substances with

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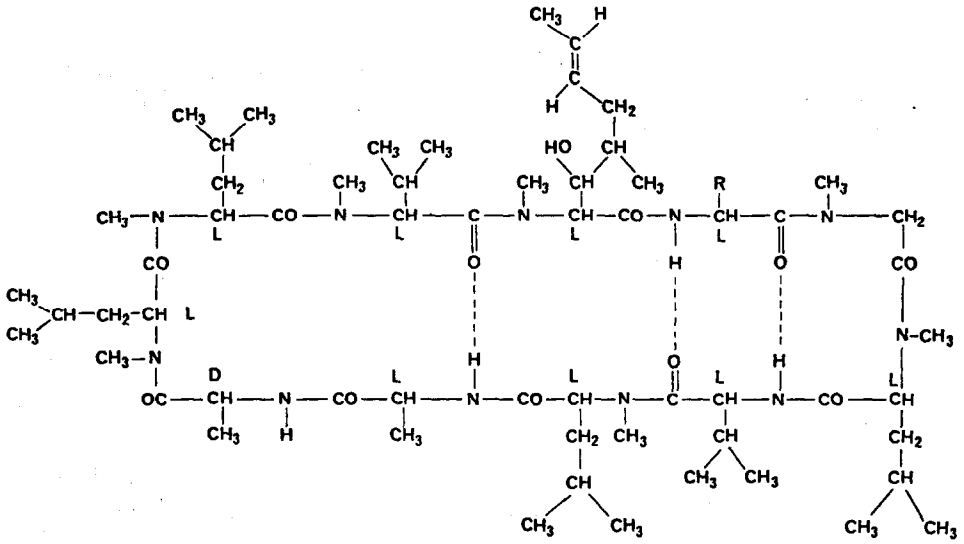

Fig. 1. Structure of cyclosporins: A, R = $-CH_2CH_3$; B, R = $-CH_3$; C, R = $-CH(CH_3)-OH$; D, R = $-CH(CH_3)_2$. L and D indicate the optical configuration of the corresponding amino acids.

nearly identical structures and higher molecular weights could be shortened. A typical example of such substances are the cyclosporins, cyclic undecapeptides (molecular weight about 1200) and metabolic products of the fungus *Tolypocladium inflatum*[6]. Cyclosporin A (Sandimmun®, Sandoz, Basle, Switzerland) has immunosuppressive properties in animals and man, and is of importance for organ transplantations[7]. Cyclosporin A is produced by fermentation[8], but other cyclosporins, such as B, C and D, are formed as well in smaller amounts.

The conventional HPLC method for the determination of cyclosporin A, B, C and D in fermentation broths[9], which requires about 12 min (10-μm particles of C_8 material), should be curtailed as far as possible with conventional instruments which also should enable an unattended analysis of about 170 samples overnight.

MATERIALS AND METHODS

Chemicals

Acetonitrile (HPLC grade S) was obtained from Rathburn Chemicals, phosphoric acid from Merck and pyrene from Fluka. The cyclosporins were from Sandoz.

Preparation of samples

One volume of fermentation broth was shaken with nine volumes of methanol for 30 min, then, filtered and diluted in ethanol–water (50:50) to a concentration of 10–100 μg/ml.

HPLC method

For the separation a stainless-steel column (15 × 0.46 cm) was slurry packed with Nucleosil C_8, 3 μm (Macherey-Nagel), and thermostatted in a Thermomix 1480

water-bath (Haake) to 70°C. The eluent was acetonitrile–water–phosphoric acid (700:300:0.1). The pump, an Altex 110 A, additionally equipped with a pulse dampener of an Orlita DMPAE pump, was set to a flow of 2 ml/min giving a back-pressure of 160 bar. The eluent was recycled. The I.D. of the capillaries was 0.25 mm, and the total length about 800 mm. The capacity of a DuPont 834 sampler was increased from 95 to 195 samples, and the loop (10 μl) of the air pressure-actuated injection valve (Valco) filled by means of a Mini S peristaltic pump (Ismatec), sucking the sample from the vials instead of applying air pressure as in the original sampler. For measurements at 214 nm a Waters M 441 detector was used (sensitivity 0.2 a.u.f.s.), the signals being recorded with a Goerz Servogor (paper advance 1 cm/min, 10 mV). The concentration of the standard solution was 50 μg each of cyclosporin A, B, C and D per ml of eluent. The data were evaluated via peak heights by means of a HP 3357 Laboratory Data System (Hewlett-Packard). After every ten samples, one standard was injected.

For detailed investigations, the usual parameters

$$N = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2; \quad H = \frac{L}{N}; \quad u = \frac{L}{t_0}$$

were evaluated where N = theoretical plate count, H = plate height, L = column length, t_0 = retention time of unretained peak at 0.5, 1.0, 2.0 and 3.0 ml/min: 156, 78, 38 and 25 sec, t_R = retention time, $w_{0.5}$ = peak width at half height and u = linear velocity. For comparison with "common" substances, a solution of pyrene (11

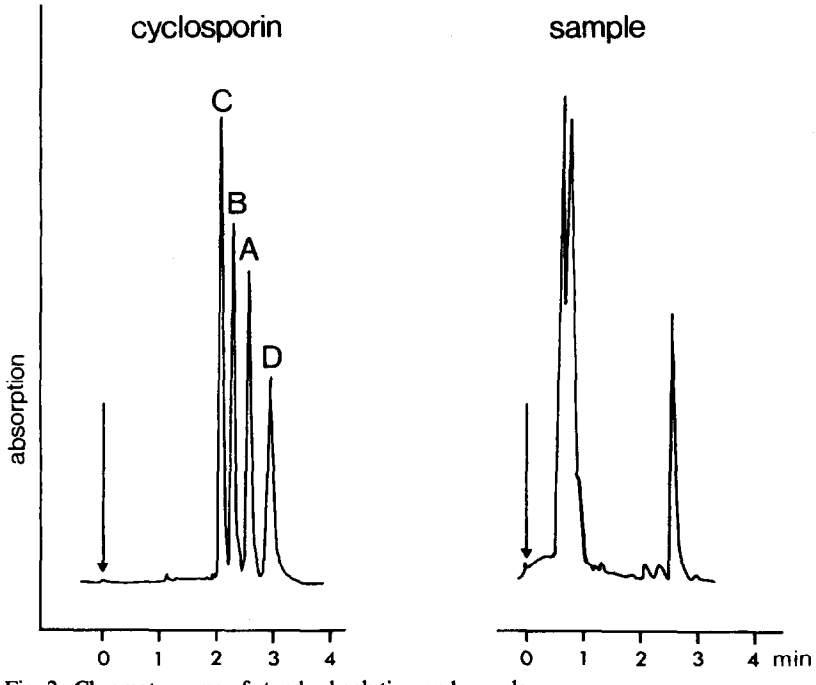

Fig. 2. Chromatograms of standard solution and sample.

μg/ml) was used. The instrumental bandwidth was determined according to DiCesare *et al.*[10].

RESULTS AND DISCUSSION

The chromatogram in Fig. 2 shows that the analysis time could be reduced from about 12 to 3.5 min. From the *H vs. u* plot (Fig. 3) it is seen that a linear velocity of 3.95 mm/sec, corresponding to a flow of 2 ml/min, is an acceptable compromise between the speed of analysis and *H*. The *H* values for cyclosporin C are higher than those for cyclosporin B, probably due to selectivity phenomena. This order is reversed on a C_{18} stationary phase.

The plate counts for pyrene are much higher than those for the cyclosporins (Table I). It can be assumed that this results from the structure and high molecular weights of these substances as it is known that a high molecular weight causes a decrease in plate count[11]. Meek and Rossetti[12] have found a good correlation between the decrease in plate count and increase in the logarithm of the molecular weight for peptides. Therefore, the gain in speed is not so drastic as is the case with small molecules. Nevertheless, the plate counts for pyrene indicate that the present instrumentation is sufficient. According to the results, a further improvement of the method is unlikely.

The column temperature of 70°C results in a low eluent viscosity, sharper peaks and minimizes or elimates temperature gradients, which could arise by viscous or frictional heating in microparticulate columns[13–16]. It should be noted that a preconditioning to 70°C of about 50 cm of the capillary before the column did not reduce peak spreading. The main reason for preferring columns with 4.6 mm I.D. instead of smaller diameters is that losses in resolution due to increasing void volumes during unattended routine analysis overnight are suppressed. A recent paper emphasizes the advantages of large-diameter (6 mm) columns[17].

The instrumental bandwidth (loop 10 μl, capillaries 40 μl, flow cell 12 μl) shows

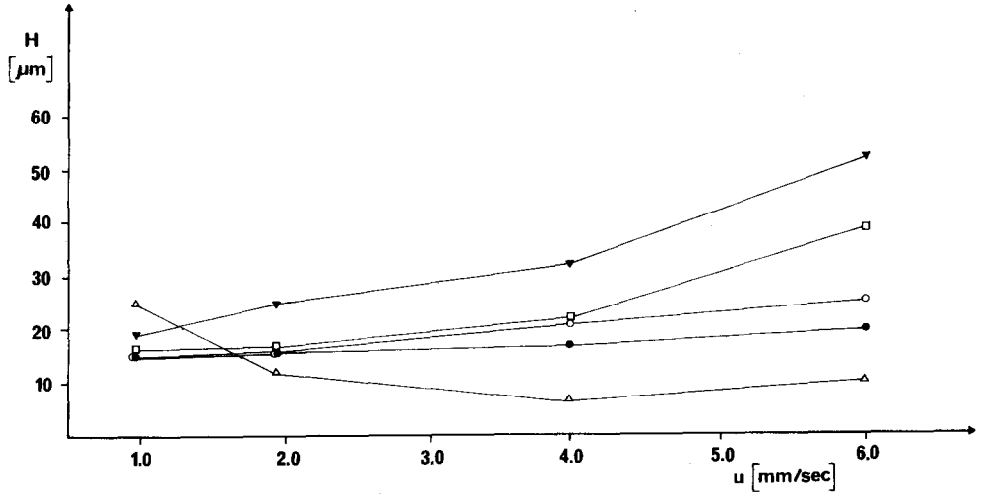

Fig. 3. H–u plots for cyclosporin A (□); B (●); C (○); D (▼); pyrene (△).

TABLE I

CHROMATOGRAPHIC DATA OF CYCLOSPORIN A, B, C AND D AND PYRENE

u = Linear velocity, t_R = retention time, H = plate height and N = theoretical plate count.

Substance	*Flow-rate (ml/min)*	*u (mm/sec)*	t_R *(sec)*	$w_{0.5}$ *(sec)*	*H (μm)*	*N*	*N per metre*
Cyclosporin C	0.5	0.96	534	12.4	15	10,274	68,495
	1.0	1.92	264	6.4	16	9427	62,844
	2.0	3.95	130	3.6	21	7224	48,162
	3.0	6.00	86	2.6	25	6061	40,408
Cyclosporin B	0.5	0.96	586	14	15	9706	64,708
	1.0	1.92	290	7	16	9508	63,390
	2.0	3.95	144	3.6	17	8864	59,093
	3.0	6.00	96	2.6	20	7553	50,352
Cyclosporin A	0.5	0.96	660	16	16	9427	62,844
	1.0	1.92	326	8.2	17	8756	58,375
	2.0	3.95	160	4.6	22	6702	44,683
	3.0	6.00	106	4	39	3890	25,936
Cyclosporin D	0.5	0.96	762	20	19	8042	53,613
	1.0	1.92	378	11.4	25	6091	40,606
	2.0	3.95	186	6.4	32	4679	31,195
	3.0	6.00	123	5.4	52	2874	19,162
Pyrene	0.5	0.96	524	8	25	5942	39,613
	1.0	1.92	260	2.7	12	12,843	85,621
	2.0	3.95	128	1	6.6	22,692	151,279
	3.0	6.00	84	0.8	9.8	15,270	101,798

a smooth decrease with increasing flow-rates (from 115 to 85 μl at 0.5 and 3.0 ml/min respectively) which is unexpected, but in accordance with the results of DiCesare *et al.*[10], and could enable —if necessary and useful— higher flow-rates. The peak width of cyclosporin A is 286 μl, the corresponding instrumental bandwidth 85 μl, so that the share of the latter is about 30%. The evaluation of the instrumental bandwidth itself should be regarded as an approximation, because the peak shape is non-Gaussian.

The coefficient of variation (N = 10) for the determination of cyclosporin A, B and C in broth samples is about 0.8%, and about 1.5% for cyclosporin D; the lifetime of the column is about 3000–5000 samples. The detection limit for cyclosporin A, B and C is about 2 μg/ml, for cyclosporin D about 3 μg/ml.

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