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Journal of Chromatography A, 925 (2001) 139–149

JOURNAL OF CHROMATOGRAPHY A

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Application of coil centrifugal counter-current chromatography to the separation of macrolide antibiotic analogues III. Effects of flow-rate, mass load and rotation speed on the peak resolution

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Received 9 March 2001; received in revised form 7 June 2001; accepted 7 June 2001

Abstract

As the third part of our methodology studies on the application of centrifugal counter-current chromatography to the preparative separation of macrolide antibiotic analogues, we have investigated the effects of various parameters on the retention of stationary phase and peak resolution. Our results show that the retention percentage of the stationary phase has linear relationships with both flow-rate at 1 to 3 ml/min and rotation speed at 100 to 700 rpm, but their correlation coefficients are negative (−1.000) and positive (0.9821), respectively. The peak resolution (R_s) is inversely proportional to the flow-rate (F_r) and mass load (M_l), but directly proportional to the rotation speed (R_{rev}). Their correlation coefficients in linear regression for the preparative separation in laboratory scale are −0.981 to −1.000 for $R_s=a+bF_r$ at flow-rates of 1 to 3 ml/min, −0.929 to −0.993 for $R_s=a+bM_l$ at mass loads of 12.5 to 100 mg, and 0.975 to 0.998 for $R_s=a+bR_{rev}$ at rotation speeds of 300 to 700 rpm, respectively. Preparative separation of six very closely related macrolide antibiotics, which belong to ascomycin and rapamycin analogues, has also been successfully achieved under optimized conditions.

Keywords: Counter-current chromatography; Flow rate; Resolution; Mass load; Preparative chromatography; Macrolides; Antibiotics

1. Introduction

Development of antibiotics necessitates isolation and purification of desired compounds from a complicated matrix such as fermentation broth and crude extract. Centrifugal counter-current chromatography (CCC) has been widely applied for the separation of natural products [1–4], including antibiotics [5]. In our purification process development, methodology on the application of CCC to the preparative separation of macrolide antibiotic analogues has been systematically evaluated. We have reported the selection of CCC solvent systems based on solubility and

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)01029-9

partition coefficient investigations [6] and the determination of partition coefficients by CCC in comparison with the shake-flask method [7]. This paper describes the effects of various parameters in preparative CCC on the retention of stationary phase and peak resolution, as well as the preparative separation of six macrolide lactones, which belong to ascomycin and rapamycin analogues.

2. Experimental

2.1. Chemicals

The macrolide compounds (Fig. 1) were obtained from Novartis Pharma (Basle, Switzerland), which include: (1) desmethylascomycin [8]; (2) ascomycin [9]; (3) FK-506 [10]; (4) dihydroFK-506 [11]; (5) rapamycin [12]; (6) DR-1, a derivative of rapamycin [40-*O*-(2-hydroxy)ethyl-rapamycin] [13]. The solvents used for high-performance liquid chromatography (HPLC) analyses were obtained from commercial sources. *tert*.-Butyl methyl ether (99.8%) was purchased from LiChrosolv (Darmstadt, Germany), acetonitrile (HPLC grade) obtained from Rathburn (Walkerburn, UK), and orthophosphoric acid (85%) was of ''Suprapur'' quality from Merck (Darmstadt, Germany). For CCC preparative separations, commercial ''HPLC grade'' *tert*.-butyl methyl ether and hexane from Fluka (Buchs, Switzerland) were used. Methanol (purity>99.9%) was obtained from Schweitzerhall (Muttenz, Switzerland). Water was purified using the Milli-Q purification system 185 from Millipore (Volketswil, Switzerland).

2.2. Apparatus

2.2.1. HPLC

A Hewlett-Packard series 1050 apparatus (Walbronn, Germany) was used for qualitative analyses.

2.2.2. CCC

A Quattro counter-current chromatography (QCCC) system (AECS, UK) [14] was equipped with a HPLC Pump 420 (Kontron, Switzerland), a Labocord 200 UV spectrophotometer (Labomatic, Switzerland) and a BR 200 recorder (Labomatic). The wavelength employed was 220 nm. A manual sample injection valve with a Rheodyne 500 μl loop (Cotati, CA, USA) was used to introduce the samples into the column. The QCCC system has four coils that are wound tightly on two separate bobbins on one rotor, each bobbin containing two concentrically wound coils. The coils are prepared by winding a long piece of PTFE tubing (1.5 mm I.D.) onto the bobbin. The total capacities of both bobbins are approximately 300 ml, respectively. The twin bobbins with the appropriate volumes maintain a balance each other, without need of counterweight. The coils

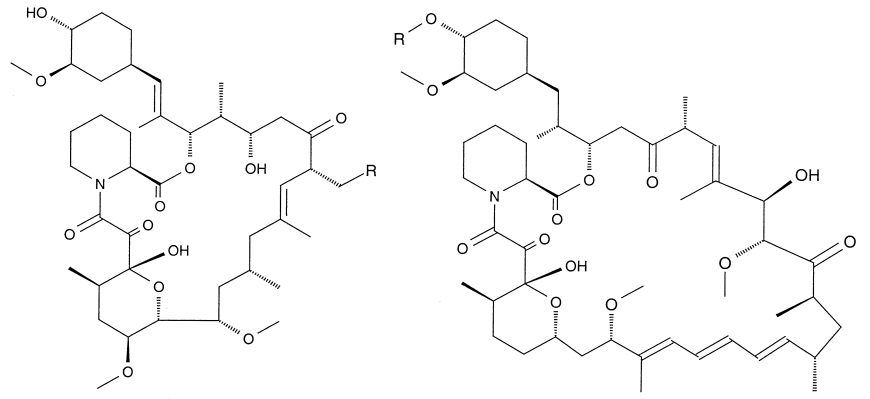


Fig. 1. Structures of the six macrolide antibiotic compounds.

are wound in pairs: 50, 250 ml on one bobbin and 100, 200 ml on the other. These coils may be interconnected in various combinations, or used one of the four independent coils. In our experiment we used two coils (50, 250 ml) on one bobbin, the total column capacity of the used coils was 300 ml. The other two coils (100, 200 ml) were filled with water for counterweight. The experimental temperature was controlled at 29±3°C with cold water. The revolution radius of the apparatus is 110 mm. The internal and outside diameters of bobbins are 126 mm and 200 mm, respectively. The minimum/maximum β values for the four coils, defined as the ratio of coil radius to orbital radius, are 0.59/0.62 for the 50 ml coil, 0.65/0.85 for the 250 ml coil, 0.59/0.67 for the 100 ml coil and 0.70/0.85 for the 200 ml coil, respectively.

2.3. Preparative separation by CCC

For preparative separation, the two-phase solvent system was prepared by continuously stirring hexane–*tert*.-butyl methyl ether–methanol–water (1:3:6:5, v/v) for 6 h. After settling at room temperature for 10 h, the solvent system was separated into organic and aqueous phases. CCC was performed on the QCCC apparatus. The used coils made a total volume capacity of 300 ml. Firstly, the column was filled with organic phase (upper layer) while the column was revolved at a designed speed. Then the aqueous phase (lower layer) was pumped through the column in a ''head to tail'' model with ''forward'' direction. Because of the poor solubility of the macrolides in both mobile and stationary phases, they were mixed in the same proportion and dissolved in 500 μl of organic phase–methanol (1:1). The eluent was continuously monitored with a UV detector at 220 nm and fractionated into test tubes (10 ml/tube) with a fraction collector. The fractions were analyzed by HPLC and compared with authentic compounds [6,7].

2.4. Measurement and calculation of parameters

The percentage retention of the stationary phase (S_f) at a given flow-rate (F_r) of the mobile phase was determined by the following procedure: the column was first entirely filled with the stationary phase. Then the mobile phase was pumped into the inlet of the column at the desired flow-rate while the apparatus was rotated at the desired speed. After the mobile phase front emerged and the two phases had established the hydrodynamic equilibrium throughout the column, the volume of the stationary phase eluted from the column was measured. The total volume of the column (V_t) includes the volume of stationary phase (V_s) and the volume of mobile phase (V_m) in the column. The percentage retention of the stationary phase (S_f) is expressed as $100V_s/(V_s+V_m)$.

Chromatographic resolution (R_s) is defined as the peak separation ($t_{R2}-t_{R1}$) divided by the average base width (W_b), which is estimated by drawing tangents to the peak inflection points and extrapolating these to the baseline: $R_s=2\cdot(t_{R2}-t_{R1})/(W_{b2}+W_{b1})$. In this equation t_R represents the peak retention time. The chromatographic efficiency, in terms of the theoretical plate number (N), is expressed as: $N=16\cdot(t_R/W_b)^2$ [2].

2.5. Statistic analysis

The single linear relationship was analyzed with Microcal Origin, Version 6.0 from Microcal Software (Northampton, MA, USA).

3. Results and discussion

Great progress with centrifugal CCC has been made in the past decades. Nevertheless, the relationship between the parameters that effect the chromatography behavior, especially in preparative CCC, has not been thoroughly studied. In our purification process development of macrolide lactone antibiotics, we investigated the effects of flow-rate and rotation speed on the retention of stationary phase, and the effects of flow-rate, mass load and rotation speed on the peak resolution in the preparative scale.

3.1. Effects of flow-rate and rotation speed on the retention of stationary phase

The retention percentage of the stationary phase (S_f) is one of the most important parameters in CCC. It is used for the derivation of the column efficiency,

peak resolution and solute retention. In a study with 15 solvent systems in three types of apparatus at various flow-rates (F_r), it was found that there is a linear relationship between the square root of the flow-rate and the retention percentage of the stationary phase (S_f) with all the correlation coefficients less than -0.992 ($S_f=a-bF_r^{1/2}$) [15]. Another regression analysis applied to the data from an aqueous two-phase solvent system using an eccentric multilayer coil planet centrifuge showed the linear relationship in the same way [16]. It has also been reported that the efficiency of CCC is much more related to the volume ratio of the mobile phase [$M_f=V_m/(V_m+V_s)$] in the column than to the flow-rate. The efficiency is not directly dependent upon the flow-rate (F_r), but increases markedly with the volume ratio of the mobile phase in the column (M_f). For each flow-rate there is a minimum value for the volume of the mobile phase, and the relationship is linear ($M_f=a+bF_r$) [17].

Employing the QCCC system manufactured by AECS (S. Wales, UK) [14], we investigated the effects of flow-rates (F_r) on the retention percentage of stationary phase (S_f) at a rotation speed of 700 rpm. Our results in Table 1 show an excellent linear relationship between the retention percentage of stationary phase and the flow-rates from 1 to 3 ml/min. The relationship can be expressed as an equation: $S_f=83-4F_r$ for the solvent system hexane–*tert*.-butyl methyl ether–methanol–water (1:3:6:5). The correlation coefficient in the regression analysis is 1.000.

At a flow-rate of 2 ml/min, the effects of rotation speed from 100 to 700 rpm on the retention percentage of stationary phase were also investigated. The results are summarized in Table 1. The linear relationship between retention percentage of stationary phase (S_f) and rotation speed (R_{rev}) was found as $S_f=38.05\ (\pm3.452)+0.056\ (\pm0.008)R_{rev.}$ The values in parentheses are the 95% confidence limits of the regression coefficients. The correlation coefficient in the regression analysis is 0.9821, the standard deviation of the linear regression 3.369 and the probability (r is zero) 0.018.

Table 1
Stationary phase retention of CCC under different conditions

R_{rev} (rpm)	F_r (ml/min)	V_s (ml)	V_m (ml)	S_f (%)	S_m (%)
700	1.0	237	63	79	21
700	2.0	225	75	75	25
700	3.0	213	87	71	29
500	2.0	202	98	67	33
300	2.0	174	126	58	42
100	2.0	124	176	41	59

Therefore, the retention percentage of stationary phase is in direct proportion to the rotation speed, but in inverse proportion to the flow-rate. Their linear relationships indicate that the retention percentage of stationary phase at certain flow-rate or rotation speed could be estimated with above two equations for the solvent system hexane–*tert*.-butyl methyl ether–methanol–water (1:3:6:5).

3.2. Effects of flow-rate, mass load and rotation speed on the peak resolution

In our investigations about the effects of flow-rate, mass load and rotation speed on the peak resolution, four mixtures were prepared with five known macrolide lactones for the preparative separation: desmethylascomycin (**1**) and ascomycin (**2**) in mixture I, ascomycin (**2**) and FK-506 (**3**) in mixture II, desmethylascomycin (**1**), ascomycin (**2**) and FK-506 (**3**) in mixture III, rapamycin (**5**) and RD-1 (**6**) in mixture IV. The structures of the selected macrolide lactones are shown in Fig. 1. These mixtures were separated by CCC with various combinations of rotation speed, flow-rate and mass load. The experimental data, including calculated theoretical plate number (N) and chromatographic resolution (R_s), are summarized in Tables 2 and 3. The results of regression analysis with Microcal Origin and their single linear relationships are presented in Table 4, in which a is the intercept value, b the slope value, r the correlation coefficient, SD the standard deviation of the linear regression, n the number of data points and P the probability that r is zero. The values in parentheses are the 95% confidence limits of the regression coefficients.

3.2.1. Effects of flow-rate (F_r) on the peak resolution (R_s)

In a previous investigation with an analytical CCC model, it was reported that a fivefold increase in flow-rate of the mobile phase resulted in a rapid separation of five components in less than 15 min without significant loss in peak resolution. The

Table 2
Preparative separation of mixtures I, II and IV by CCC

	F_r (ml/min)	M_l (mg)	R_{rev} (rpm)	t_{R1}	W_{b1} (min)	N_1	t_{R2} (min)	W_{b2} (min)	N_2	R_s
Mixture II				Desmethylascomycin (**1**)			Ascomycin (**2**)			
	1	12.5	700	215	27	1015	264	32	1089	1.66
	1	25.0	700	212	30	799	261	33	1001	1.56
	1	50.0	700	209	36	539	258	38	738	1.32
	1	75.0	700	200	37	467	250	46	473	1.20
	2	12.5	700	110	16	756	134	19	796	1.37
	2	25.0	700	109	18	587	133	19	784	1.30
	2	50.0	700	109	20	475	133	22	585	1.14
	2	75.0	700	106	20	449	129	23	503	1.07
	3	12.5	700	77	11	784	91	14	676	1.12
	3	25.0	700	76	13	547	91	15	589	1.07
	3	50.0	700	76	15	411	90	14	661	0.97
	3	75.0	700	74	15	389	88	16	484	0.90
Mixture II				Ascomycin (**2**)			FK-506 (**3**)			
	1	12.5	700	263	35	903	313	36	1209	1.41
	1	25.0	700	250	34	865	298	42	805	1.26
	1	50.0	700	270	39	767	317	47	728	1.09
	1	75.0	700	256	43	567	305	51	572	1.04
	2	12.5	700	146	22	705	172	22	978	1.18
	2	25.0	700	143	20	818	164	21	976	1.02
	2	50.0	700	141	23	601	161	23	784	0.87
	2	75.0	700	141	26	471	161	24	720	0.80
	3	12.5	700	94	13	837	106	16	702	0.83
	3	25.0	700	92	16	529	106	18	555	0.82
	3	50.0	700	91	19	367	105	20	441	0.72
	3	75.0	700	95	20	361	109	21	431	0.68
Mixture IV				Rapamycin (**5**)			DR-1 (**6**)			
	2	75	700	197	32	606	151	24	633	1.64
	2	100	700	200	34	554	155	25	615	1.53
	2	150	700	202	34	565	155	28	490	1.52
	2	200	700	203	34	570	154	28	484	1.58
	2	75	500	184	33	497	146	28	435	1.25
	2	100	500	187	34	484	149	29	422	1.21
	2	150	500	188	36	436	150	30	400	1.15
	2	200	500	188	40	353	148	33	322	1.10
	2	75	300	171	28	597	147	22	714	0.96
	2	100	300	176	29	589	150	25	576	0.96
	2	150	300	174	32	473	149	24	617	0.89
	2	200	300	145	27	409	1	27	461	0.89

retention values were decreased while the maximum pressure was increased. The retention of stationary phase also tends to decline with the increased flow-rate, but in a much slower rate [18]. In another study about the discrepancy between the theoretical plate number and peak resolution for optimizing the flow-rate in CCC, the mobile phase at 0.5–8.0 ml/min has been applied. The results showed that the theoretical plate number is probably not always a reliable measure in CCC separation, since R_s represents the actual separation of the solute peaks [19].

Within the maximum possible pressure of the

Table 3
Preparative separation of mixture III by CCC at a rotation speed of 700 rpm

F_r (ml/min)	M_l (mg)	Desmethylascomycin (**1**)			Ascomycin (**2**)			FK-506 (**3**)			$R_{s(1-2)}$	$R_{s(2-3)}$
		t_{R1}	W_{b1}	N_1	t_{R2}	W_{b2}	N_2	t_{r3}	W_{b3}	N_3		
1.0	33.3	210	30	784	260	36	835	308	45	750	1.52	1.19
1.0	50.0	210	32	689	260	41	643	308	49	632	1.37	1.07
1.0	75.0	214	33	673	259	45	530	304	53	526	1.15	0.92
1.0	100.0	230	45	418	278	47	560	320	56	522	1.04	0.82
2.0	33.3	119	20	566	144	26	491	168	24	784	1.09	0.96
2.0	50.0	120	21	522	146	30	379	170	26	684	1.02	0.86
2.0	75.0	120	23	436	144	30	369	168	31	470	0.91	0.79
2.0	100.0	114	25	333	139	32	302	163	33	390	0.88	0.74
3.0	33.3	82	15	478	97	19	417	112	17	694	0.88	0.83
3.0	50.0	84	17	391	99	20	392	114	20	520	0.81	0.75
3.0	75.0	84	18	348	100	23	302	115	22	437	0.78	0.67
3.0	100.0	89	17	439	103	20	424	117	24	380	0.76	0.64

applied QCCC apparatus, we could increase the flow-rate from 1 to 3 ml/min in our experiments. The correlation between flow-rate and peak resolution was investigated by separating desmethylascomycin (**1**), ascomycin (**2**) and FK-506 (**3**) in different mixtures. By comparing the experimental data of mixtures I, II and III in Tables 2 and 3, it can be easily found that both peak retention time (t_R) and average peak base width (W_b) were clearly decreased while flow-rate (F_r) was increased. Moreover, the peak resolution (R_s) was also decreased accordingly in all cases (Fig. 2).

As presented in Table 4, the statistic analysis of our experimental data shows significant single linear relationship between peak resolution and flow-rate from 1 to 3 ml/min. The correlation coefficients in the regression analyses at different mass loads are between -0.981 and -1.000. The relationship between peak resolution and flow-rate is expressed as $R_s = a + bF_r$. In this equation, the slope value (b) represents the decreasing of the peak resolution resulted from higher flow-rate. By comparing b values of all three mixtures at different mass loads, it can also be found that the decreasing of the peak resolution resulted from higher flow-rate is in inverse proportion to the mass load.

3.2.2. Effects of mass load (M_l) on peak resolution (R_s)

In CCC, unlike any solid stationary phase, the whole liquid stationary phase is available for solute exchange. Therefore, it was believed that its efficiency remains remarkably constant when solutes in high concentration were injected [20]. Mass overload will occur only when the liquid stationary phase is saturated by the solute. However, the increase of injection volume produced a decrease in separation efficiency. As in traditional liquid chromatography, larger injection volume produces a band broadening, which is particularly important for the less retained solutes [20]. It was also reported that increasing injected amount of hop extract results in tailing and fronting. The elution profiles are more Gaussian, and the maxima are flattened. Larger injection volumes result in broader peaks and more fronting and tailing, but no effect on retention or run time was observed [21].

In our investigations about the effects of mass load on the peak resolution, we separated mixtures I, II and III with the same injection volume of 500 μl. The mass loads are ranged from 12.5 to 75 mg for mixtures I and II, and from 33.3 to 100 mg for mixture III. The experimental data in Tables 2 and 3 show that higher mass load (M_l) results in lower peak resolution (R_s) and broader average peak base width (W_b), but has almost no effect on peak retention time (t_R).

The experimental data are presented in Fig. 3. In our statistic analysis (Table 4), the single linear relationship between peak resolution and mass load is expressed as $R_s = a + bM_l$. Correlation coefficients in the regression analyses at different mass loads are

Table 4
Results of linear regression analyses

Compound	M_l (mg)	F_r (ml/min)	M_l (mg)	a	b	r	SD	n	P
$R_s=a+bF_r$									
1–**2** in mixture I	12.5			1.923 (±0.025)	−0.270 (±0.012)	−0.999	0.016	3	0.027
	25.0			1.800 (±0.019)	−0.245 (±0.009)	−0.999	0.012	3	0.022
	50.0			1.493 (±0.006)	−0.175 (±0.003)	−1.000	0.004	3	0.011
	75.0			1.357 (±0.025)	−0.150 (±0.012)	−0.997	0.016	3	0.049
2–**3** in mixture II	12.5			1.720 (±0.075)	−0.290 (±0.035)	−0.993	0.049	3	0.076
	25.0			1.473 (±0.025)	−0.220 (±0.012)	−0.999	0.016	3	0.033
	50.0			1.263 (±0.044)	−0.185 (±0.020)	−0.994	0.029	3	0.069
	75.0			1.200 (±0.075)	−0.180 (±0.035)	−0.982	0.049	3	0.121
1–**2** in mixture III	33.3			1.803 (±0.137)	−0.320 (±0.064)	−0.981	0.090	3	0.125
	50.0			1.627 (±0.087)	−0.280 (±0.040)	−0.990	0.057	3	0.091
	75.0			1.317 (±0.069)	−0.185 (±0.032)	−0.986	0.045	3	0.108
	100.0			1.173 (±0.025)	−0.140 (±0.012)	−0.997	0.016	3	0.052
2–3 in mixture III	33.3			1.353 (±0.062)	−0.180 (±0.029)	−0.987	0.041	3	0.101
	50.0			1.213 (±0.062)	−0.160 (±0.029)	−0.984	0.041	3	0.114
	75.0			1.043 (±0.006)	−0.125 (±0.003)	−1.000	0.004	3	0.015
	100.0			0.913 (±0.012)	−0.090 (±0.006)	−0.998	0.008	3	0.041
$R_s=a+bM_l$									
1–**2** in mixture I		1		1.742 (±0.037)	−0.008 (±0.001)	−0.990	0.037	4	0.010
		2		1.421 (±0.028)	−0.005 (±0.001)	−0.986	0.029	4	0.014
		3		1.159 (±0.010)	−0.004 (±0.000)	−0.996	0.010	4	0.004
2–**3** in mixture II		1		1.436 (±0.061)	−0.006 (±0.001)	−0.953	0.062	4	0.047
		2		1.205 (±0.057)	−0.006 (±0.001)	−0.960	0.058	4	0.040
		3		0.869 (±0.018)	−0.003 (±0.000)	−0.979	0.019	4	0.021
1–**2** in mixture III		1		1.744 (±0.056)	−0.007 (±0.001)	−0.988	0.041	4	0.012
		2		1.185 (±0.039)	−0.003 (±0.001)	−0.972	0.028	4	0.028
		3		0.916 (±0.033)	−0.002 (±0.001)	−0.929	0.024	4	0.071
2–**3** in mixture III		1		1.359 (±0.034)	−0.006 (±0.001)	−0.993	0.024	4	0.007
		2		1.043 (±0.038)	−0.003 (±0.001)	−0.971	0.028	4	0.029
		3		0.906 (±0.037)	−0.003 (±0.001)	−0.967	0.026	4	0.033
$R_s=a+bR_{rev}$									
5–**6** in mixture IV			75	0.433 (±0.076)	0.002 (±0.000)	0.996	0.041	3	0.054
			100	0.521 (±0.053)	0.001 (±0.000)	0.998	0.029	3	0.045
			150	0.399 (±0.084)	0.002 (±0.000)	0.995	0.045	3	0.064
			200	0.328 (±0.205)	0.002 (±0.000)	0.975	0.110	3	0.141

between −0.929 and −0.993 at mass loads of 12.5 to 100 mg. In the relationship equation, the slope value (b) represents the decreasing of peak resolution resulted from higher mass load. By comparing b values of all three mixtures at different mass loads, it can be found that decreasing of the peak resolution resulted from higher mass load is in inverse proportion to the flow-rate.

3.2.3. Effects of rotation speed (R_{rev}) on peak resolution (R_s)

In a report on preparative scale operation of cephalosporin C and desacetyl cephalosporin C by CCC in an aqueous two-phase system, several rotational speeds and flow-rates were tried, and their effects on the number of theoretical plate and retention were measured. Contrary to what had been

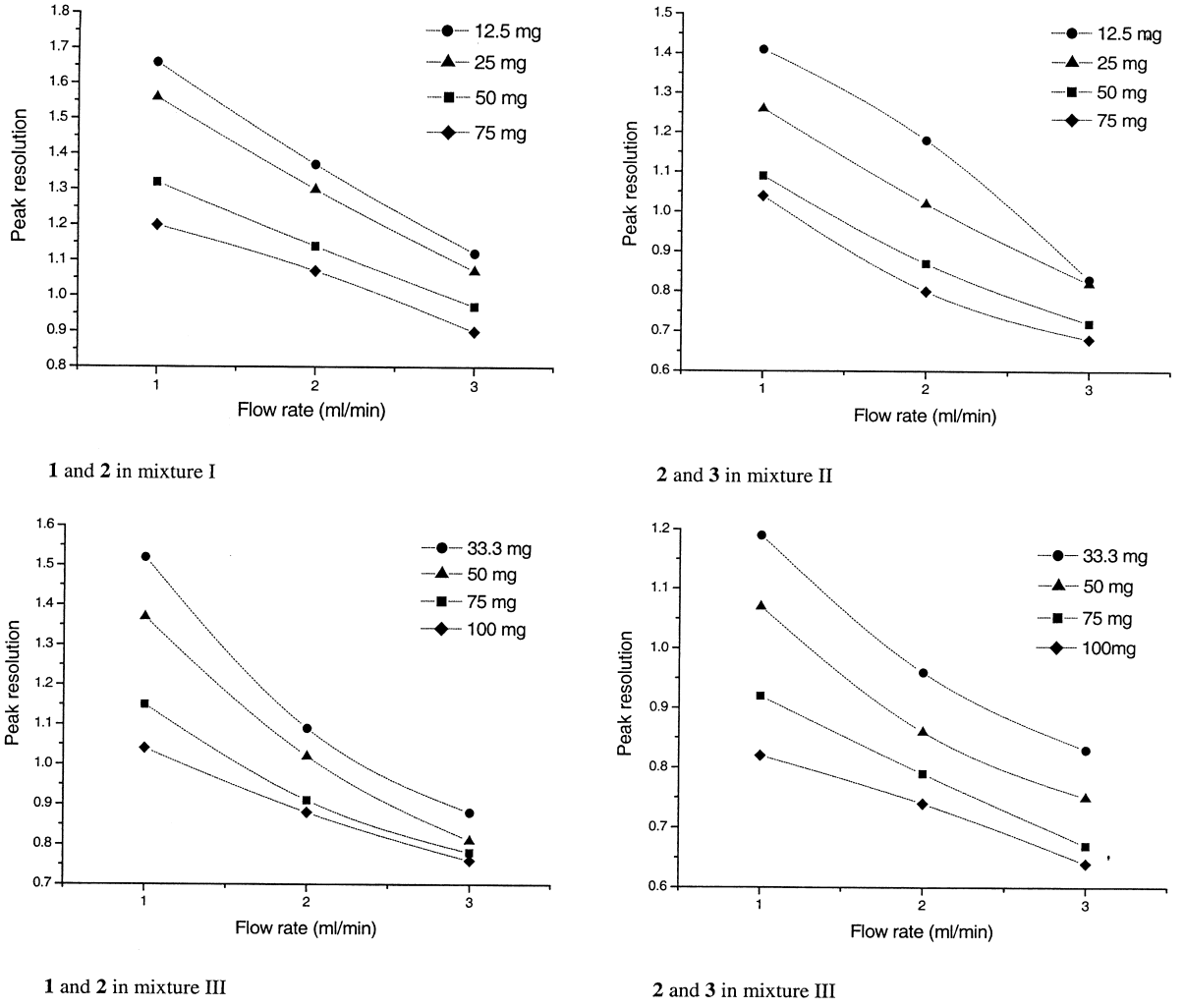


Fig. 2. Correlation between peak resolution (R_s) and flow-rate (F_r) at various mass loads (M_l).

observed in the coaxial type of columns, high rotation speeds lowered the retention ratio at the range of 300–800 rpm. Number of theoretical plate increased with rotational speed but retention decreased simultaneously [22].

At a flow-rate of 2 ml/min, we separated rapamycin (**5**) and DR-1 (**6**) in mixture IV at various rotation speeds. The experimental data are summarized in Table 2. The correlation between peak resolution and rotation speed is presented in Fig. 4. At higher rotation speed, both peak resolution (R_s) and retention time (t_R) were clearly increased. The single linear relationship between peak resolution and rotation speed is expressed as $R_s = a + bR_{rev}$ in Table 4. Correlation coefficients in the regression analysis at different rotation speeds are between 0.975 and 0.998 at mass load of 300 to 700 rpm. Slope value (b) in the relationship equation, which represents the increasing of the peak resolution resulted from higher rotation speed, is not proportional to the mass load.

3.3. Preparative separation of six macrolides by CCC under optimized conditions

Our investigations clearly show that higher rota-

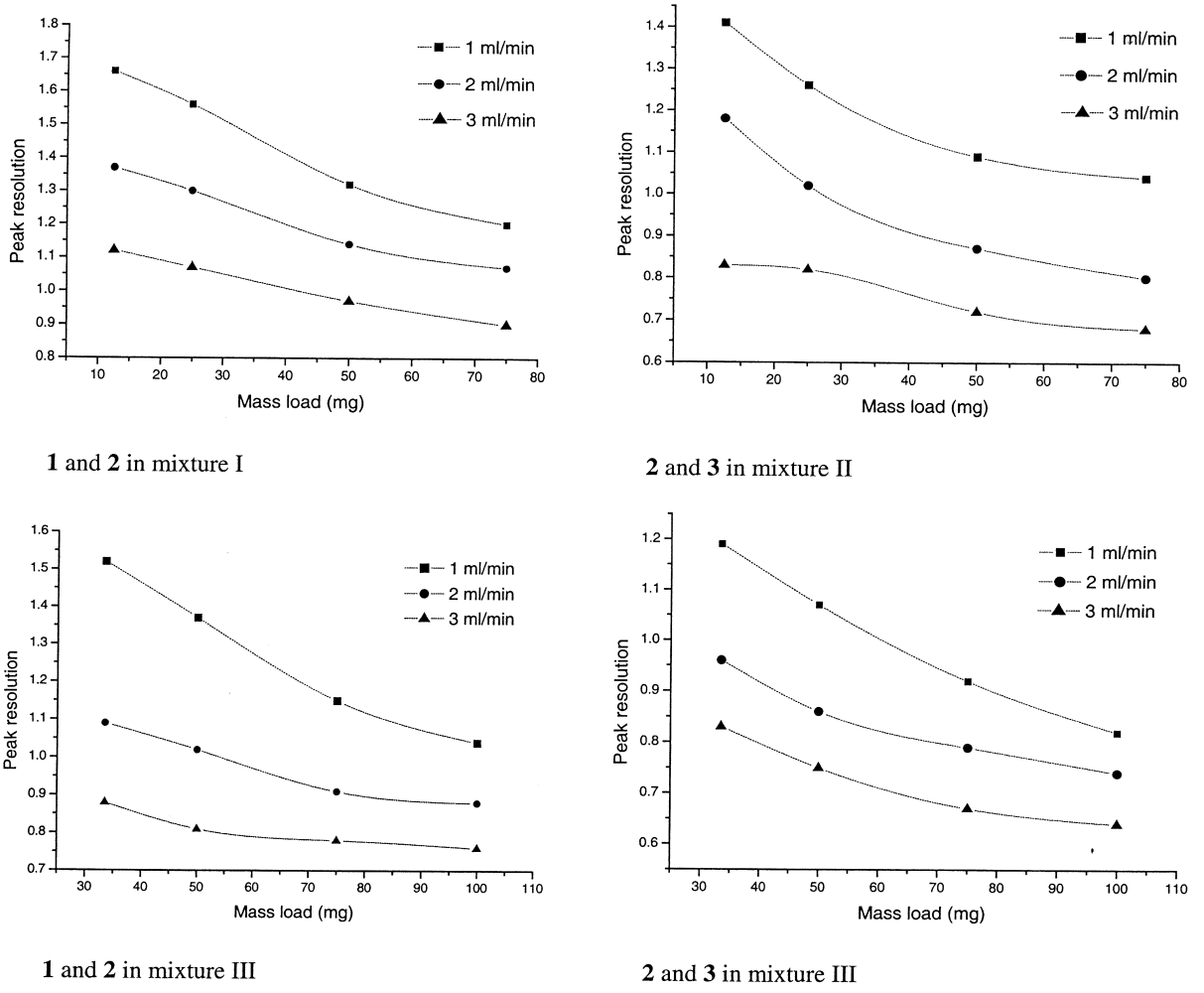


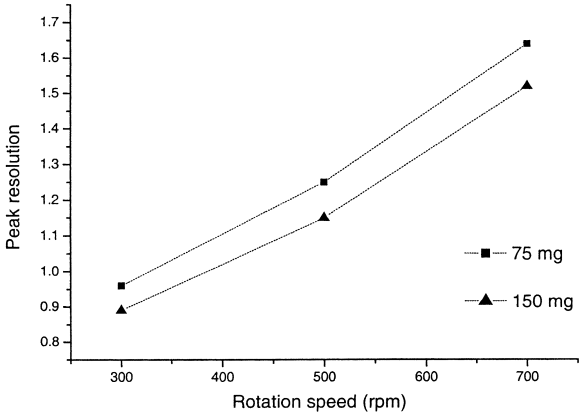
Fig. 3. Correlation between peak resolution (R_s) and mass load (M_l) at various flow-rates (F_r).

tion and lower flow-rate can increase the peak resolution. Therefore, we have selected the flow-rate at 1 ml/min and the rotation speed at 700 rpm to separate six macrolide antibiotic analogues, including desmethylascomycin (**1**), ascomycin (**2**) and FK-506 (**3**) in mixture III, rapamycin (**5**) and DR-1 (**6**) in mixture IV, desmethylascomycin (**1**), ascomycin (**2**), FK-506 (**3**) and dihydroFK-506 (**4**) in mixture V. The preparative scales are 50 and 75 mg for mixture III, 100 mg for mixture IV and 25 mg for mixture V, respectively. All separations with the quaternary solvent system (hexane–*tert*.-butyl methyl ether–methanol–water, 1:3:6:5) were quite successful and their chromatograms are shown in Fig. 5.

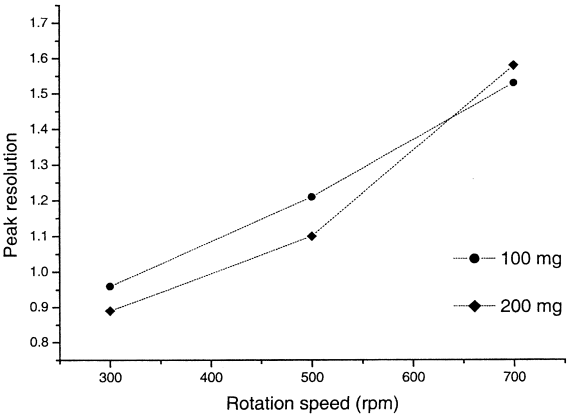
4. Conclusions

As the third part of our methodology studies on the application of CCC to the preparative separation of macrolide antibiotic analogues, we have investigated the effects of various parameters on the retention of stationary phase and peak resolution.

In our approach, we have tried to simplify the complicated relationships among various factors and in different combinations by linear regression analysis for the limited narrow range in preparative CCC. Although this simplification might be inapplicable to the extended CCC application, it is really very helpful to find some practical guide for the estima-

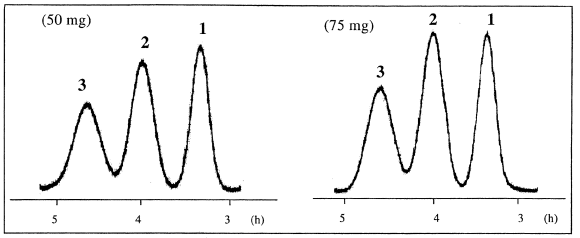


5 and **6** in mixture IV at mass loads 75 and 150 mg

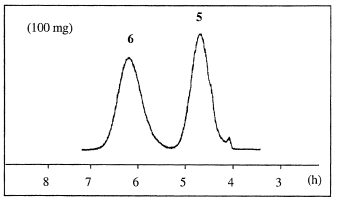


5 and **6** in mixture IV at mass loads 100 and 200 mg

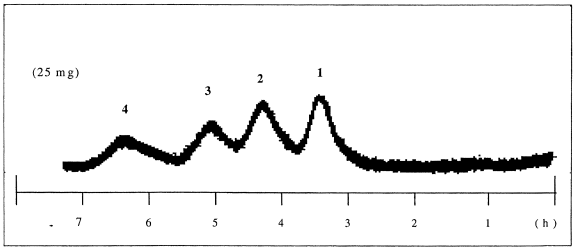
Fig. 4. Correlation between peak resolution (R_s) and rotation speed (R_{rev}) at various mass loads (M_1).



(a) Preparative separation of mixture III by CCC



(b) Preparative separation of mixture IV by CCC



(c) Preparative separation of mixture V by CCC

1 desmethylascomycin, **2** ascomycin, **3** FK-506, **4** dihydroFK-506, **5** rapamycin, **6** DR-1

Fig. 5. Preparative separation of six macrolide antibiotic compounds by CCC.

tion of suitable parameters and for the prediction of experimental results in preparative CCC optimization. In the meanwhile, it could be also conveniently used to compare the effects of various parameters, such as the slope value in the relationship equations. Due to the technical limit of applied equipment, only a few of points in each set of data could be evaluated in our experiments and used for statistic analysis. The data formed exponential trends in many cases, but they may be approximately expressed in linear equations within limited ranges.

Our results show that the retention percentage of stationary phase in preparative CCC has linear relationships with both flow-rate at 1 to 3 ml/min and rotation speed at 100 to 700 rpm, but their correlation coefficients are negative and positive, respectively. The peak resolution (R_s) is inversely proportional to the flow-rate from 1 to 3 ml/min and to the mass load from 12.5 to 100 mg, but directly proportional to the rotation speed from 300 to 700 rpm. All their relationships are linear in our regression analysis. Preparative separation of six very closely related macrolide antibiotics with CCC has also been successfully achieved under optimized conditions, which is based on our study about various factors of importance for the quality of a preparative separation.

References

[1] K. Hostettmann, M. Hostettmann, A. Marston, Preparative Chromatography Techniques, Applications in Natural Product Isolation, Springer, Berlin, Heidelberg, 1986.
[2] W.D. Conway, Countercurrent Chromatography – Apparatus, Theory, and Applications, VCH, New York, 1990.
[3] A. Marston, K. Hostettmann, J. Chromatogr. A 658 (1994) 315.
[4] A.P. Foucault, L. Chevolot, J. Chromatogr. A 808 (1998) 3.
[5] H. Oka, K. Harada, Y. Ito, Y. Ito, J. Chromatogr. A 812 (1998) 35.
[6] W. Wang-Fan, E. Küsters, O. Lohse, C. Mak, Y. Wang, J. Chromatogr. A 864 (1999) 69.
[7] W. Wang-Fan, E. Küsters, C. Mak, Y. Wang, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 1365.
[8] A.B. Smith, K.J. Hale, L.M. Laakso, K. Chen, A. Riera, Tetrahedron Lett. 30 (1989) 6963.
[9] H. Hatanaka, T. Kino, S. Miyata, N. Inamura, A. Kuroda, T. Goto, H. Tanaka, M. Okuhara, J. Antibiot. 41 (1988) 1592.
[10] T. Kino, H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, J. Antibiot. 40 (1987) 1249.
[11] T. Hirokazu, K. Akio, M. Hiroshi, H. Hiroshi, K. Toru, G. Toshio, H. Masashi, T. Toru, J. Am. Chem. Soc. 109 (1987) 5031.
[12] S.N. Sehgal, H. Baker, C. Vezina, J. Antibiot. 28 (1975) 727.
[13] S. Cottens, R. Sedrani, PCT Int. Appl. (1994) WO 9409010 A1 19940428.
[14] I.A. Sutherland, L. Brown, S. Forbes, G. Games, D. Hawes, K. Hostettmann, E.H. McKerrell, A. Marston, D. Wheatley, P. Wood, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 279.
[15] Q. Du, C. Wu, C. Qian, P. Wu, Y. Ito, J. Chromatogr. A 835 (1999) 231.
[16] L. Xia, J. Hsu, J. Liq. Chromatogr. 15 (1992) 2801.
[17] A.P. Foucault, O. Bousquet, F.L. Goffic, J. Cazes, J. Liq. Chromatogr. 15 (1992) 2721.
[18] T. Zhang, L.K. Pannell, Y. Ito, J. Chromatogr. 445 (1988) 199.
[19] H. Oka, Y. Ikai, J. Hayakawa, K. Harada, K. Nagase, M. Suzuki, H. Nakazawa, Y. Ito, J. Liq. Chromatogr. 15 (1992) 2707.
[20] A. Berthod, D.W. Armstrong, J. Liq. Chromatogr. 11 (1988) 1187.
[21] A.C.J. Hermans-Lokkerbol, A.C. Hoek, R. Verpoorte, J. Chormatogr. A 771 (1997) 71.
[22] P. Lin, I. Chu, Biotechnol. Techn. 9 (1995) 549.