Application of centrifugal counter-current chromatography to the separation of macrolide antibiotic analogues
I. Selection of solvent systems based on solubility and partition coefficient investigations

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Abstract

As the first part of our studies on counter-current chromatography, the methodology for selecting suitable solvent systems was established based on detailed investigations of solubility and partition coefficients (log K) of macrolide antibiotic analogues. The solubility of two important macrolides, ascomycin and FK-506, was measured in a series of common solvents, where their polarities were ranked with dielectric constants. The partition coefficients of the two macrolides were compared in various binary, ternary, quaternary solvent systems. Hexane–*tert*.-butyl methyl ether–methanol–water system was selected based on suitable log K of solutes and hydrogen-bonding properties of solvents. In the further optimisation of composition proportions in the multicomponent solvent system, hexane–*tert*.-butyl methyl ether–methanol–water (1:3:6:5) showed the best solvent selectivity by giving the most prominent difference of partition coefficient (Δlog K) between ascomycin and FK-506. With this solvent system, a baseline preparative separation of these two very closely related 23-membered macrolide antibiotics was successfully achieved by employing the newly introduced Quattro counter-current chromatograph.   © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

Centrifugal counter-current chromatography (CCC), also known as centrifugal partition chromatography (CPC) and high-speed counter-current chromatography (HSCCC), has been widely used for separation of both natural and synthetic products [1–3]. This solid support free liquid–liquid partition chromatographic method efficiently eliminates troubles resulted by conventional solid support of liquid chromatography, such as irreversible adsorption or degradation of compounds. The separation of compounds by CCC is based on the difference in partitioning behaviour of solutes between the two immiscible liquid phases. The separated constituents

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are eluted normally in the order of the respective partition coefficients.

Antibiotics are normally biosynthesised as mixtures of closely related congeners and many of them are labile molecules, thus requiring mild separation techniques with a high resolution capacity. Therefore, CCC has also been associated with the field of antibiotics since its inception and is becoming a powerful tool to separate components from antibiotics complexes [4]. Various antibiotics with different structure types and carbon skeletons have been isolated and purified by CCC [2–4]. Macrolides are an important antibiotic family and many of them demonstrate very potent biological activities. Only a few macrolides have been isolated or purified by CCC, such as 36-membered RS-22A, B and C [5], 2-norerythromycin A, B, C and D [6]. In our purification process development, we have evaluated and investigated methodology on the application of CCC to the preparative separation of macrolide antibiotic analogues, including important immunosuppressive agents ascomycin, FK-506, rapamycin and their derivatives. This paper describes the selection of solvent systems based on solubility and partition coefficient studies, as well as the preparative separation of two very closely related 23-membered macrolide lactones, ascomycin and FK-506.

2. Experimental

2.1. Chemicals

The solvents used in measuring solubility of ascomycin and FK-506 covered a wide range of polarity, including water, methanol, ethanol, acetone, ethyl acetate, tert.-butyl methyl ether, cyclohexane, hexane, n-heptane, etc. The solvents were of quality "purissimum" or analytical grade and obtained from Fluka (Buchs, Switzerland) and Aldrich (Steinheim, Germany). The solvents used for high-performance liquid chromatography (HPLC) analyses: tert.-butyl methyl ether (99.8%) LiChrosolv grade from Merck (Darmstadt, Germany), acetonitrile (HPLC grade) was obtained from Rathburn (Walkerburn, UK), and orthophosphoric acid (85%) was of "Suprapur" quality from Merck. Water was purified using the Milli-Q purification system 185 from Millipore

(Volketswil, Switzerland). Ascomycin and FK-506 used in the experiments were obtained from Novartis Pharma (Basle, Switzerland). For CCC preparative separations, commercial "HPLC grade" tert.-butyl methyl ether and hexane from Fluka were used. Methanol (purity>99.9%) was obtained from Schweitzerhall (Muttenz, Switzerland).

2.2. Apparatus

For HPLC a Hewlett-Packard series 1050 apparatus (Waldbronn, Germany) was used for quantitative analyses. For CCC a Quattro counter-current chromatograph (QCCC) (AECS, UK) was equipped with a HPLC Pump 420 (Kontron, Switzerland), a Labocord-200 UV spectrophotometer (Labomatic, Switzerland), a BR 200 recorder (Labomatic) and a Labocol Vario 10 fraction collector (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 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 coils are wound in pairs: 50, 250 ml on one bobbin and 100, 200 ml on the other. In our experiment the total column capacity of the used coils was 300 ml. The other two coils (100, 200 ml) were filled with water as counterweight. The experiment temperature was controlled at $29 \pm 3°C$ with cold water. The revolution speed of the rotor was adjusted to 700 rpm. The revolution radius of the apparatus (R) is 110 mm. The internal and outside diameter of bobbins is 126 mm and 200 mm, respectively. The minimum/maximum β values for the four coils, defined as the ratio of coil radius (r) to orbital radius (R), are 0.587/0.616 for the 50 ml coil, 0.645/0.847 for 250 ml coil, 0.587/0.674 for the 100 ml coil and 0.703/0.847 for the 200 ml coil, respectively.

2.3. Quantitative HPLC analysis

For quantitative measurement of ascomycin, a Nucleosil 120-5 C_{18} column (250×4 mm I.D.) (Macherey-Nagel, Oensinger, Switzerland) was applied at the temperature of 40°C. An elution com-

posed of 78% of solution A (*tert*.-butyl methyl ether–acetonitrile–water–orthophosphoric acid, 6:29:65:0.02) and 22% of solution B (*tert*.-butyl methyl ether–acetonitrile–water–orthophosphoric acid, 13.6:66.4:20:0.02) was employed with a flow-rate of 1.5 ml/min. FK-506 was quantitatively analysed with a Nucleosil 100-5 C_{18} AB column (250×4 mm I.D.) (Macherey-Nagel) at a temperature of 60°C. The solution of *tert*.-butyl methyl ether–acetonitrile–water–orthophosphoric acid (8.65:42.1:49.25:0.01) was used with a flow-rate of 1.0 ml/min. Both ascomycin and FK-506 were detected with a UV detector at 210 nm.

2.4. Measurement of solubility

The solubility was defined as the maximum amount (g) of solute dissolved in 100 ml of solvent at definite temperature. The saturated solutions of both ascomycin and FK-506 were prepared at room temperature (25±1°C) by dissolving solutes in solvents with continuously stirring until saturation. The amounts of solutes in saturated solutions were quantitatively analysed by HPLC after being properly diluted with methanol–water (9: 1) for ascomycin and with acetonitrile for FK-506.

2.5. Measurement of partition coefficients (log K)

The partition coefficients (log K) of both ascomycin and FK-506 were measured with the "shake-flask" method. Various solvent systems of samples were prepared by continuously stirring solutions at constant room temperature (25±1°C) for 30 min before separating into organic and aqueous phases. To measure the partition coefficient, the sample solution (50 μg/ml) was prepared with organic (or aqueous) phase, and the solution gently shaken with an equal volume of aqueous (or organic) phase for 3 min, then settled at room temperature for 2 h. The organic and aqueous phases were separated. The concentrations of solute in two phases were quantitatively analysed by HPLC, respectively. As the quantitative measurement is conducted by HPLC, log K may be calculated as follows:

$$\log K = \log [C]_{org} - \log [C]_{aq}$$
$$= \log A_{org} - \log A_{aq} \tag{1}$$

where log K is partition coefficient; $[C]_{org}$ and $[C]_{aq}$ are the concentrations of solute in organic and aqueous phases, respectively; A_{org} and A_{aq} are the adsorbance peak areas of solute in organic and aqueous phases measured by HPLC, respectively.

2.6. Preparative separation by CCC

For separating ascomycin and FK-506 by CCC, a two-phase solvent system was prepared by continuously stirring hexane–*tert*.-butyl methyl ether–methanol–water (1:3:6:5) for 6 h. After settling at room temperature for 10 h, the solvent system was separated into organic and aqueous phases. The QCCC column was filled with organic phase (upper layer) while the column was revolved at 700 rpm. Then the aqueous phase (lower layer) was pumped through the column in a "head to tail" (H→T) model with "forward" direction. The flow was 1 ml/min during the rotation. Due to the poor solubility of ascomycin and FK-506 in both mobile and stationary phases, the mixture of ascomycin and FK-506 (1:1, 25 mg) was dissolved in 500 μl of organic phase–methanol (1:1). The eluent was continuously monitored at 220 nm and fractionated into test tubes (10 ml/tube).

3. Results and discussion

Two important 23-membered macrolide analogues, ascomycin and FK-506 (Fig. 1), have been selected as the representatives to start our investigation. FK-506 is a macrolide antibiotic, identified from *Streptomyces tsukubaensis* [7–9]. Ascomycin, isolated from *Streptomyces hygroscopicus* subsp. *yakushimaensis*, is an analogue of FK-506 class [10]. In 1987, FK-506 was reported as one of the most active immunosuppressants [11–13]. Due to its much higher potency than cyclosporin A (CsA) [14,15], many medical [16,17], biological [18,19], biochemical [20,21], and synthetic [22–24] investigations have been made on this type of macrolides. Therefore, the purification development of these antibiotic analogues is of interest not only for macrolide separation technique, but also for new drug development.
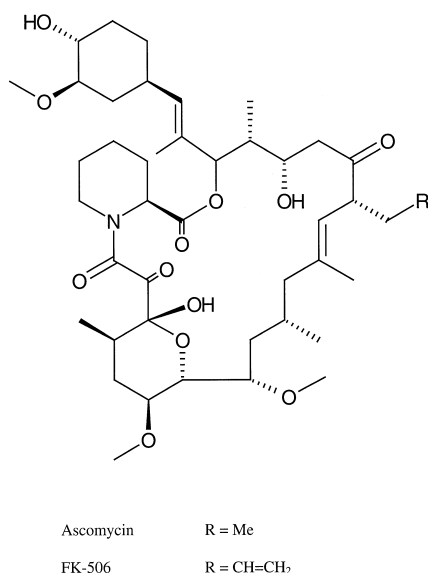
| Ascomycin | R = Me |
| FK-506 | R = CH=CH₂ |

Fig. 1. Structures of FK-506 and ascomycin.

3.1. Solubility studies and selection of solvents

There is no doubt that successful CCC depends on the correct choice of solvent systems, because the separation of compounds by CCC technique is mainly based on the difference in partitioning behaviour of solutes between the two immiscible liquid phases. Partitioning involves distribution of the solute between two phases. However, as a general rule, the ability of a solvent to extract the solutes parallels its capacity to dissolve the solutes. Therefore, as one of the most important physicochemical properties of the solutes related to CCC, their solubility in various solvents should be first quantitatively analysed.

The term ''polarity'' is a vague designation used in solvent extraction and chromatography to designate the rank-order ability of solvents to extract or increase the rate of chromatographic migration of a particular solute. Solvent polarity is a key factor to determine the approximate magnitude of the partition coefficient observed for a particular compound, which in turn determines the retention time in chromatography. The dielectric constant (ϵ) offers one of the simplest means of ranking solvents in order of polarity. A series of common solvents, with the dielectric constants ranging from 1.89 (hexane)

to 78.54 (water), were used to measure the solubility of ascomycin and FK-506. Although chloroform-based systems are most frequently employed in CCC due to their large density differences and relatively high interfacial tensions between the two solvent phases, none of solvents containing halogen was selected considering the important ecological factor in our purification process development. The experimental results on solubility studies are summarised in Table 1, together with the dielectric constants (ϵ) excerpted from literatures [25,26]. As can be found from Table 1, the solubility of ascomycin and FK-506 could be grouped under three categories: very low solubility (<0.1 g/100 ml) in extremely polar solvent (water) or extremely apolar solvents (hexane, cyclohexane and n-heptane), moderate solubility (2–3 g/100 ml) in medium polar solvent (tert.-butyl methyl ether) and high solubility (>10 g/100 ml) in polar solvents (methanol, ethanol and acetone) and apolar solvent (ethyl acetate). This information could be used as the base for solvents selection in biphasic multicomponent solvent systems.

3.2. Preliminary selection of the solvent systems based on partition coefficients and hydrogen-bonding properties

Selection of the composition of solvent systems for chromatography can be guided by several means [2,3,27]. An empirical approach is solvent screening by thin-layer chromatography (TLC), where an organic layer of a two-phase aqueous system is used

Table 1
Solubility of ascomycin and FK-506 in various solvents with different polarities (at 25°C)

Solvent	ϵ (20°C)	Solubility (g/100 ml)	
		Ascomycin	FK-506
Water	78.54	0.037	0.074
Methanol	32.63	24.95	15.40
Ethanol	24.30	12.11	11.99
Acetone	20.70	26.61	28.37
Ethyl acetate	6.02	18.64	17.65
tert.-Butyl methyl ether	4.50	2.850	3.770
Cyclohexane	2.02	0.045	0.050
Hexane	1.89	0.042	0.008
n-Heptane	1.92	0.006	0.015

as the eluent and the R_f values are the selection criteria for both normal-phase and reversed-phase modes [28]. This method is very simple and convenient, but gives only an approximate indication of the utility of a particular solvent, because TLC involves both partition and adsorption mechanisms, whereas CCC is based on purely liquid–liquid partition phenomena. The use of rapid analytical CCC is also a valid method for the choice of solvent systems for preparative-scale separation. However, care has to be taken while differences between the analytical and preparative instrumentation and the viscosity of solvents may lead to variations [3].

Measuring partition coefficients is another mean to find a suitable solvent. Considering the separation principles of CCC, we have applied this method for getting more accurate information on screening solvent systems. The solutes are partitioned between two immiscible liquid phases with classic "shake-flask" method and their respective concentrations are measured by reversed-phase HPLC. Both ascomycin and FK-506 belong to the same 23-membered macrolides type and there is only very minor difference in the structures. Therefore, their general partition in various biphasic solvent systems must be very similar. In order to save considerable time, only the partition coefficients of ascomycin have been measured in multicomponent solvent systems for the preliminary selection.

A series of binary, ternary, quaternary solvent systems were prepared based on the solubility studies. Binary solvent systems are seldom used because of the large difference in polarity between the two components. Therefore, we have put our emphasis on ternary and quaternary solvent systems, in which the addition of a third or fourth component, miscible with the other components, diminishes the difference in polarity between the two phases and increases the selectivity of the solvent system for closely-related substances.

In our experiments, the partition coefficients of ascomycin in 15 solvent systems were determined (Table 2). Both theory and practice suggest that solvent systems yielding partition coefficients of 0.2 to 5 (log K between -0.69 and 0.6), or over perhaps a slightly broader range, are most useful in CCC [2]. As can be found in Table 2, the partition coefficients of two ternary (T5 and T7) and three quaternary (Q1, Q4 and Q5) solvent systems are exactly in this range.

From another aspect, partition behaviour of solutes is always associated with various interactions in the

Table 2
Partition coefficients of ascomycin in various solvent systems (at 25°C)

	Solvent system	Proportion	K	log K
Binary system				
B1	*n*-Heptane–methanol	1:1	0.014	-1.86
Ternary systems				
T1	*n*-Heptane–methanol–water	5:4:1	0.003	-2.58
T2	*n*-Heptane–methanol–water	10:7:3	0.005	-2.29
T3	*n*-Heptane–isopropanol–water	1:1:1	0.081	-1.09
T4	*n*-Heptane–isopropanol–water	1:2:1	0.052	-1.28
T5	*n*-Heptane–isopropanol–water	1:1:2	0.372	-0.43
T6	*n*-Heptane–isopropanol–water	2:1:1	0.095	-1.02
T7	*n*-Heptane–acetone–water	1:1:1	0.479	-0.32
T8	*tert*.-Butyl methyl ether–methanol–water	2:1:1	4.677	0.67
T9	*tert*.-Butyl methyl ether–methanol–water	5:2:3	30.903	1.49
Quaternary systems				
Q1	Hexane–ethyl acetate–methanol–water	8:2:10:5	2.188	0.34
Q2	Hexane–ethyl acetate–methanol–water	1:9:5:10	74.131	1.87
Q3	Hexane–*tert*.-butyl methyl ether–methanol–water	1:5:4:5	10.233	1.01
Q4	Hexane–*tert*.-butyl methyl ether–methanol–water	1:4:5:5	2.455	0.39
Q5	Hexane–*tert*.-butyl methyl ether–methanol–water	1:3:6:5	0.550	-0.26

system (e.g., solute–solute, solute–solvent, solvent–solvent) [29–31]. One of the most important effects is the hydrogen-bonding, which is directly related to solvent selectivity. The molecule of ascomycin contains multiple functional groups that serve as hydrogen donors (e.g., hydroxyl groups) and acceptors (e.g., methoxyl and ketone groups). Thus, it can be expected to interact much strongly with hydrogen-acceptor and hydrogen-donor solvents. Such specific interactions play an important role in determining the solvent selectivity in chromatography. In the quaternary solvent system of hexane–*tert*.-butyl methyl ether–methanol–water, the aqueous and organic phases contain mainly methanol and water, hexane and *tert*.-butyl methyl ether, respectively. According to Hecker's solvent classification [32], water and methanol belong to amphibious solvents, which are both hydrogen-acceptors and hydrogen-donors. Hexane is inert and *tert*.-butyl methyl ether is a hydrogen-bond acceptor. Therefore, such a quaternary solvent system should offer better selectivity and a more suitable and flexible medium for partitioning of ascomycin than all the ternary solvent systems, where the organic phase is mainly composed of only inert solvent.

3.3. Optimisation of component proportions of preliminarily selected quaternary solvent system by comparing Δ log K between ascomycin and FK-506

Separation of two compounds in chromatography is commonly expressed by the chromatographic resolution R_s in Eq. (2), where t is the retention time and W_b is the base width of chromatographic peaks [2].

$$R_s = \frac{2(t_2 - t_1)}{W_{b1} + W_{b2}} = \frac{2\Delta t}{W_{b1} + W_{b2}} \qquad (2)$$

Starting from the definition equation of resolution, an exact resolution equation for CCC was derived and presented by Conway and Ito [33] in the same format as the Knox equation [34]. The exact resolution equation summarised major determinative of resolution in CCC, including separation factor term, efficiency term and exact partition coefficient term.

$$R_s = \frac{1}{4}(\alpha - 1)\sqrt{N}$$

$$\times \left\{ \frac{K_1}{K_1[(\alpha + 1)/2] + [(1 - S_F)/S_F]} \right\} \qquad (3)$$

In Eq. (3), α is the separation factor, N the number of theoretical plates, K the partition coefficient, and S_F the fractional volume of column occupied by stationary phase. The separation factor α, which is defined as the ratio of the partition coefficients of two solutes, is directly related to solvent system selectivity and makes the greatest contribution to resolution in CCC. Therefore, the partition coefficients of both ascomycin and FK-506 have been measured in the preliminarily selected quaternary solvent systems with various component proportions in order to maximise the separation factor α. The difference of log K between ascomycin and FK-506, defined as Δ log K, is calculated together with separation factor α. As can be easily seen in Table 3, hexane–*tert*.-butyl methyl ether–methanol–water in the proportion of 1:3:6:5 gives the most prominent difference of partition coefficients Δ log K (0.149) between ascomycin and FK-506, namely the highest value of separation factor α (1.409). Therefore, it should be the best solvent system selectivity for ascomycin and FK-506, and could make a great contribution to achieving higher resolution in CCC. In addition, the volume ratio of this biphasical system is also in a rational range.

3.4. Preparative separation of ascomycin and FK-506 by CCC with selected quaternary solvent system in optimised component proportion

Two types of CCC instruments are commercially available, one with rotating coils consisting of wrapped PTFE tubes and another with rotating cartridges containing separation channels. Various centrifugal counter-current chromatographs have been manufactured by different companies [2,3]. A new addition, the QCCC manufactured recently by AECS was used in our investigations. A novel feature of this new instrument is the absence of a central shaft which allows the four coils to have their maximum possible radius. The four coils may be used independently or

Table 3
Partition coefficients and separation factors of ascomycin and FK-506 in various solvent systems (at 25°C)

Solvent system (v/v)	Ascomycin		FK-506		$\Delta \log K$[b]	α[c]
	K[a]	$\log K$[a]	K[a]	$\log K$[a]		
Hexane–tert.-butyl methyl ether–methanol–water (1.75:5:3.25:5)	18.493	1.267	20.989	1.322	0.055	1.135
Hexane–tert.-butyl methyl ether–methanol–water (2:5:3:5)	17.660	1.247	16.520	1.218	0.029	1.069
Hexane–tert.-butyl methyl ether–methanol–water (3:5:3:5)	6.412	0.807	5.916	0.772	0.035	1.084
Hexane–tert.-butyl methyl ether–methanol–water (4:5:3:5)	5.848	0.767	6.067	0.783	0.016	1.038
Hexane–tert.-butyl methyl ether–methanol–water (5:5:3:5)	3.793	0.579	3.899	0.591	0.012	1.028
Hexane–tert.-butyl methyl ether–methanol–water (1:5:4:5)	10.162	1.007	10.568	1.024	0.017	1.040
Hexane–tert.-butyl methyl ether–methanol–water (1:4:5:5)	2.477	0.394	3.342	0.524	0.130	1.349
Hexane–tert.-butyl methyl ether–methanol–water (1:3:6:5)	0.545	−0.264	0.767	−0.115	0.149	1.409

[a] Partition coefficient of solute measured with "shake-flask" method, the quantitative analyses carried out by HPLC.

[b] Absolute difference between partition coefficient values of ascomycin and FK-506.

[c] Separation factor defined as the ratio of the partition coefficients of two solutes and always expressed as a value greater than unity.

interconnected in various combinations to satisfied different needs. The ability to maintain sub-ambient temperature also increase its application to thermally
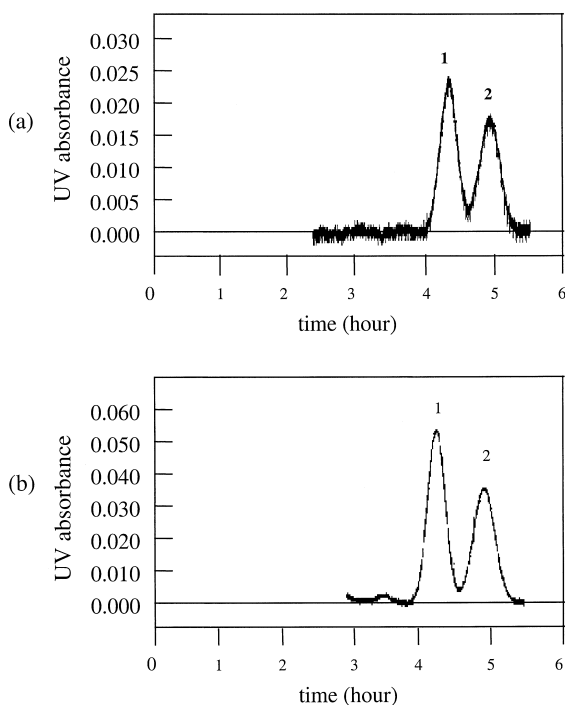

Fig. 2. Preparative separation of ascomycin and FK-506 by CCC: (a) ascomycin and FK-506 (1:1): 25 mg; (b) ascomycin and FK-506 (1:1): 50 mg. Peaks 1 and 2 correspond to ascomycin and FK-506, respectively. CCC with hexane–tert.-butyl methyl ether–methanol–water (1:3:6:5): coil volume 300 ml; the volume of stationary phase in coils 238 ml, H→T model, 700 rpm, 1 ml/min, 220 nm.

labile compounds and with more volatile solvents. Furthermore, the temperature control can also keep physical and physicochemical properties of both solvents and solutes in relatively stable state, such as viscosity, density, solubility and partition coefficient etc., increasing the reproducibility of CCC.

In general, either phase in the biphasical solvent system may be employed as the stationary phase in CCC. This depends, to great extent, on the value of the partition coefficients of solutes in the system. Considering the relatively smaller $\log K$ value of ascomycin (−0.264) and FK-506 (−0.115) in hexane–tert.-butyl methyl ether–methanol–water (1:3:6:5), we have selected the reversed-phase mode for their separation, i.e., organic stationary phase and aqueous mobile phase in CCC. The mixture of ascomycin and FK-506, in amounts of 25 mg and 50 mg, respectively, was successfully separated with CCC. Their chromatograms are shown in Fig. 2, in which peaks 1 and 2, confirmed by HPLC analysis, correspond to ascomycin and FK-506, respectively.

4. Conclusions

As the first part of our methodology studies on the application of centrifugal CCC to preparative separation of macrolide antibiotic analogues, we have developed a practical and efficient method for selecting a solvent system based on solubility and partition coefficient investigations. Exemplified with two important immunosupressive macrolides, ascomycin

and FK-506, a quaternary solvent system (hexane–*tert*.-butyl methyl ether–methanol–water, 1:3:6:5) was selected and optimised according to the physicochemical properties of both solutes and solvents. Employing the newly introduced QCCC, a baseline preparative separation of these two very closely related 23-membered macrolide antibiotics was achieved with selected solvent system.

References

[1] K. Hostettmann, M. Hostettmann, A. Marston, Preparative Chromatography Techniques, Applications in Natural Product Isolation, Springer Verlag, 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] H. Oka, K. Harada, Y. Ito, Y. Ito, J. Chromatogr. A 812 (1998) 35.

[5] M. Ubukata, N. Shiraishi, K. Kobinata, T. Kudo, I. Yamaguchi, H. Osada, Y. Shen, K. Isono, J. Antibiot. 48 (1995) 289.

[6] R.H. Chen, J.E. Hchlowski, J.B. McAlpine, R.R. Rasmussen, J. Liq. Chromatogr. 11 (1988) 191.

[7] O. Masakuni, T. Hirokazu, G. Toshio, K. Tohru, H. Hiroshi, Eur. Pat. Appl., 123 pp. EP 184162 A2 860611.

[8] T. Kino, H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, J. Antibiot. 40 (1987) 1249.

[9] H. Tanaka, A. Kuroda, H. Marusawa, H. Hatanaka, T. Kino, T. Goto, M. Hashimoto, J. Am. Chem. Soc. 109 (1987) 5031.

[10] H. Hatanaka, T. Kino, S. Miyata, N. Inamura, A. Kuroda, T. Goto, H. Tanaka, M. Okuhara, J. Antibiot. 41 (1988) 1592.

[11] T. Ochiai, K. Nakajima, M. Nagata, T. Suzuki, T. Asano, T. Uematsu, T. Goto, S. Hori, T. Kenmochi, T. Nakagoori, K. Isono, Transplant. Proc. 19 (1987) 1284.

[12] S. Sawada, G. Suzuki, Y. Kawase, F. Takaku, J. Immunol. 139 (1987) 1797.

[13] T. Kino, H. Hatanaka, S. Miyata, N. Inamura, M. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, T. Ochiai, J. Antibiot. 40 (1987) 1256.

[14] K.L. Napoli, J. Int. Fed. Clin. Chem. 4 (1992) 15.

[15] S.L. Schreiber, Science 251 (1991) 283.

[16] T. Ochiai, K. Nakajima, M. Nagata, S. Hori, T. Asano, K. Isono, Transplantation 44 (1987) 734.

[17] A. Ito, T. Ito, W. Kamiike, A. Moriguchi, A. Ohkawa, F. Uchikoshi, S. Tanaka, S. Nakata, H. Matsuda, Transplantation 64 (1997) 752.

[18] A.P. Weetman, in: A.P. Weetman, A. Grossman (Eds.), Pharmacotherapeutics of the Thyroid Gland, Springer-Verlag, Berlin, Heidelberg, 1997, p. 349, Ch. 14.

[19] M.W. Harding, A. Galat, D.E. Uehling, S.L. Schreiber, Nature 341 (1989) 758.

[20] J. Liu, W. Albers, T.J. Wandless, S. Luan, D.G. Alberg, P.J. Belshaw, P. Coven, C. mackintosh, C.B. Klee, S.L. Schreiber, Biochemistry 31 (1992) 3896.

[21] H.M. Organ, M.A. Holmes, J.M. Pisano, M.J. Staruch, M.J. Wyvratt, F.J. Dumont, P.J. Sinclair, Bioorg. Med. Chem. Lett. 3 (1993) 657.

[22] T.K. Jones, S.G. Mills, R.A. Reamer, D. Askin, R. Desmond, R.P. Volante, I. Shinkai, J. Am. Chem. Soc. 111 (1989) 1157.

[23] D.R. Williams, J.W. Benbow, J. Org. Chem. 53 (1988) 4643.

[24] A.B. Smith III, K.J. Hale, L.M. Laakso, K. Chen, A. Riéra, Tetrahedron Lett. 30 (1989) 6963.

[25] D.R. Lide (Editor-in-Chief), CRC Handbook of Chemistry and Physics, CRC Press, Boca Raton, FL, 71st ed., 1990–1991.

[26] C. Reichardt, in: Solvents and Solvents Effects in Organic Chemistry, VCH, Weinheim, 1988, p. 408.

[27] A.P. Foucault, L. Chevolot, J. Chromatogr. A 808 (1998) 3.

[28] K. Hostettmann, Adv. Chromatogr. 21 (1983) 165.

[29] W. Fan, N. El Tayar, B. Testa, L.B. Kier, J. Phys. Chem. 94 (1990) 4764.

[30] W. Fan, R. Tsai, N. El Tayar, P. Carrupt, B. Testa, J. Phys. Chem. 98 (1994) 329.

[31] R. Tsai, W. Fan, N. El Tayar, P. Carrupt, B. Testa, L.B. Kier, J. Am. Chem. Soc. 115 (1993) 9632.

[32] E. Hecker, Verteilungsverfahren in Laboratorium, Verlag Chemie, Weinheim, 195, p. 102.

[33] W.D. Conway, Y. Ito, J. Liq. Chromatogr. 8 (1985) 2195.

[34] A.S. Said, Theory and Mathematics of Chromatography, Hüthig, New York, 1981.