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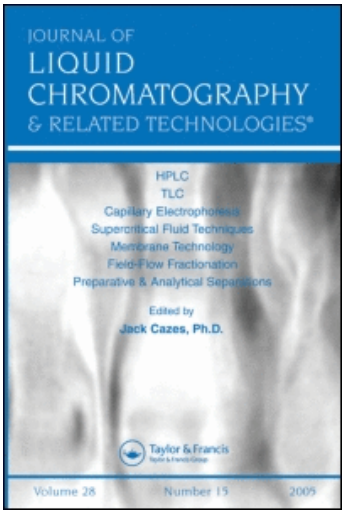

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Weizheng Wang-Fan[a]; Ernst Küsters[a]; Ching -Pong Mak[a]; Ying Wang[a]
[a] Novartis Pharma Inc., Basle, Switzerland

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APPLICATION OF CENTRIFUGAL COUNTER-CURRENT CHROMATOGRAPHY TO THE SEPARATION OF MACROLIDE ANTIBIOTIC ANALOGUES. II. DETERMINATION OF PARTITION COEFFICIENTS IN COMPARISON WITH THE SHAKE-FLASK METHOD

Weizheng Wang-Fan,[1] Ernst Küsters,[1,*] Ching-Pong Mak,[1] Ying Wang[2]

[1] Chemical and Analytical Development

[2] Core Technology Area
Novartis Pharma Inc.
CH-4002 Basle, Switzerland

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ABSTRACT

The separation of compounds by centrifugal counter-current chromatography (CCC) is based on the difference in partition behaviour of solutes between the two immiscible liquid phases. Therefore, Δlog K, defined as the difference of partition coefficients of the solutes, is a key criterion in the solvent system selection for the CCC. Partition coefficients (log K) of macrolide antibiotic analogues can be measured by different ways, such as direct shake-flask method or indirect HPLC analysis. However, there are also some clear limitations and shortcomings with these methods. As an alternative, a Quattro Counter-Current Chromatograph was examined to measure log K for a series of macrolide antibiotic analogues and compared with the classical shake-flask method. The experimental results shows that partition coefficients measured by CCC are highly correlated with those determined by shake-flask method. The major advantages of the CCC method are rapidity, precision, and the possibility to

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determine log K of analytes in mixtures. Therefore, CCC is a promising alternative to directly measure the partition coefficients of macrolide antibiotics analogues in purification process development.

INTRODUCTION

Centrifugal counter-current chromatography (CCC) is a continuous liquid-liquid partition method without any solid supporting matrix. In a CCC system, one phase remains by a high revolutional speed which provides the necessary centrifugal force for sustaining the stationary phase, while the second phase is pumped through the system as mobile phase.[1] The separation of compounds by a CCC technique is based on the difference in partitioning behaviour of solutes between the two immiscible liquid phases. As described in our previous paper of this series, Δlog K, defined as the difference of partition, is a key criterion in the solvent system selection for CCC.[2] It is also one of the most important parameters in physicochemical property evaluations of macrolide antibiotic analogues.

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Traditionally, the shake-flask method is a classical and direct experimental way for measuring partition coefficients (log K).[3, 4] This method, however, is not suitable for mixtures and for the compounds with log K values higher than 3. The experimental process is also difficult to be standardized and automated. The determination of partition coefficients by HPLC analysis is an indirect way, in which either a solid stationary phase or a liquid stationary phase fixed on an "inert" solid support is used. Although chromatographic log k' values of analytes are linearly correlated to log K values of the compounds,[5,6] the interactions between solutes and solid supports or packing materials in HPLC technique are significantly different from the solute-solvent interactions in the shake-flask method.[7-9] Droplet counter-current chromatography (DCCC), lack of interactions with a solid packing material and with similar principles to the shake-flask method, has been adapted to log K determinations.[10] It is based on the partitioning of solutes between a steady stream of droplets of mobile phase and a column of surrounding stationary phase.[11] The major shortcomings of DCCC method are long running time and the necessity to choose solvent systems which form droplets. Centrifugal counter-current chromatography (CCC), also known as high-speed counter-current chromatography (HSCCC) or centrifugal partition chromatography (CPC), has also been employed for measuring partition coefficients with small amounts of samples in a variety of solvent systems.[12-14]

Macrolides are an important antibiotic family and many of them demonstrate very potent biological activities.[15] In our purification process development, we have evaluated and investigated methodology on the application of CCC to the preparative separation of macrolide antibiotic analogues.[2] In this

paper, we report the log K measurement by CCC with a series of macrolide antibiotic analogues, in comparison with the classical shake-flask method.

EXPERIMENTAL

Chemicals

The solvents used for HPLC analyses: tert-butylmethyl ether (99.8%) was purchased from LiChrosolv (Darmstadt, Germany), acetonitrile (HPLC grade) obtained from RATHBURN (Walkerburn, Scotland), and ortho-phosphoric acid (85%) was of "Suprapur" quality from Merck (Darmstadt, Germany). Water was purified using the Milli-Q purification system 185 from Millipore (Volketswil, Switzerland). The macrolide compounds were obtained from Novartis Pharma Inc. (Basle, Switzerland): (1) FK-506,[16] (2) ascomycin,[17] (3) desmethylascomycin,[18] (4) DA-1, a derivative of ascomycin (8-ethyl-3,4,5,6,8,11,12,13,14,15,16,17,18,19,22,23,24,25-octadecahydro -5,15,19-trihy-droxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethenyl] -14,16-dimeth-oxy-4,10,12,18-tetramethyl-1H-19,25a-methanopyrido [2,1-c][1,4]oxaazacyclo docosine-1,7,20,26-tetrone),[19] (5) rapamycin,[20] (6) DR-1, a derivative of rapamycin (40-O-(2-hydroxy)ethyl-rapamycin),[21] (7) DR-2, a derivative of rapamycin (27-deoxorapamycin).[22] The solvents used for CCC: tert-butylmethyl ether and hexane (HPLC grade) were purchased from Fluka (Buchs, Switzerland), methanol (purity > 99.9%) was obtained from Chem. Farb. Schweitzerhall (Muttenz, Switzerland).

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Apparatus

A Hewlett-Packard series 1050 HPLC apparatus (Walbronn, Germany) was used for quantitative analyses. A Quattro Counter Current Chromatograph (QCCC)(AECS, UK) was equipped with an HPLC PUMP 420 (KONTRON, Zurich, Switzerland), a LABOCORD-200 UV-spectrophotometer (LABOMATIC, Switzerland), a BR 200 recorder (LABOMATIC, Switzerland), and a LABOCOL Vario 10 fraction collector (LABOMATIC, Switzerland). The wavelength employed was 220 nm. A manual sample injection valve with a Rheodyne 500 ∝L loop (COTATI, U.S.A.) was used to introduce the samples into the column. In our experiments with QCCC, we used two coils (50, 250 mL) on one bobbin, the other two coils (100, 200 mL) were filled with water as counterweight. The rotational speed of the rotor was adjusted to 700-800 rpm.

Quantitative HPLC Analysis

For quantitative analysis of FK-506 and desmethylascomycin, a Nucleosil 100 - 5 C18 AB column (250 x 4 mm i.d.) (Macherey-Nagel, Oensinger,

Switzerland) was employed at 60°C. Isocratic elution with tert-butylmethyl ether - acetonitrile - water - ortho-phosphoric acid (8.65 : 42.1 : 49.25 : 0.01) was used with a flow-rate of 1.0 mL/min.

The amounts of ascomycin were quantitatively analysed with a Nucleosil 120 - 5 C18 column (250 x 4 mm i.d.) (Macherey-Nagel, Oensinger, Switzerland) at 40°C and detected at 210 nm. Solution A (78%, tert-butylmethyl ether - acetonitrile - water - ortho-phosphoric acid 6 : 29 : 65 : 0.02) and solution B (22%, tert-butylmethyl ether - acetonitrile - water - other-phosphoric acid 13.6 : 66.4 : 20: 0.02) was employed for a isocratic elution with a flow-rate of 1.5 mL/min.

The quantitative analysis of DA-1 was performed with a packed ODS column (250 x 4.6 mm i.d.) at 55°C. The mobile phase composed of 55% of solution A (water - acetonitrile 9 : 1) and 45% of solution B (water - acetonitrile 2 : 8) was isocratically eluted at a flow-rate of 1.0 mL/min.

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Rapamycin and DR-1 were analysed with a Nucleosil 120-3 C 18 column (250 x 4 mm i.d.) (Macherey-Nagel, Oensinger, Switzerland) at 50°C. A gradient elution was employed with methanol-water (96:2 to 100:0 in 5 min. and continued with 100% methanol for another 10 min.). The flow-rate was 0.9 mL/min.

For analysis of DR-2, a Nucleosil 120-3 C 18 column (250 x 4 mm i.d.) (Macherey-Nagel, Oensinger, Switzerland) was used at the temperature of 60°C. The elution composed of methanol - water (7 : 3) was isocratically eluted at a flow-rate of 0.6 mL/min.

Measurement of Partition Coefficients (log K) with the Shake-Flask Method

The partition coefficients (log K) of the macrolide compounds were measured with the classical shake-flask. The two-phase solvent system was prepared by continuously stirring hexane, tert-butylmethyl ether, methanol, and water in the proportion of 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 sample solution (~25 ∝g/mL) was prepared with organic (or aqueous) phase, and gently shaken with an equal volume of aqueous (or organic) phase for 3 min. After settling at room temperature for 2 h, organic and aqueous phases were separated. The concentrations of solute in both phases were quantitatively analysed by HPLC. The partition coefficient (log K) of solute was calculated with the following equation:

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

where $[C]_{org}$ and $[C]_{aq}$ are the concentrations of solute in organic and aqueous phase, respectively; A_{org} and A_{aq} are the HPLC peak areas of solute in organic and aqueous phase, respectively.

Measurement of Partition Coefficients (log K) with the CCC Method

Due to the poor solubility in both mobile and stationary phases, each macrolide (~25 mg) was dissolved in 500 ∝l of organic phase - methanol (1 : 1). Hexane - tert-butylmethyl ether - methanol - water (1 : 3 : 6 : 5) was prepared in the same way as described above. The descending mode was applied with a Quattro Counter Current Chromatograph (QCCC). The coils were initially filled with organic phase (upper layer) as the stationary phase while the coils were revolved at 700-800 rpm. Then the aqueous phase (lower layer) was pumped through the column in a "head to tail" model with "forward" direction. The flow-rate of mobile phase was controlled at 1 mL/min during the rotation. The partition coefficients were calculated with following equation:

$$\log K = \log \frac{V_r - V_m}{V_t - V_m} \qquad (2)$$

where V_r is the retention volume of an analyte, V_m is the mobile phase volume in the system and V_t is the total volume of the system.[23]

Statistic Analysis

The single linear relationship between partition coefficients measured by CCC [log $K_{(CCC)}$] and by shake-flask method [log $K_{(SF)}$] was analysed with Microcal™ Origin™, Version 4.1 from Microcal Software Inc. (Northampton, USA).

RESULTS AND DISCUSSION

In our purification process development of macrolide antibiotics analogues, seven compounds belonging to FK-506 or rapamycin class, were selected. Four of them are microbial metabolites isolated from various strains of *Streptomyces*. The other three are synthetic derivatives of macrolides. Their structures and sources are summarised and presented in Figure 1 and Table 1, respectively. The partition coefficients of these macrolides have been measured in hexane - tert-butylmethyl ether - methanol - water (1 : 3 : 6 : 5) with both CCC and shake-flask method. The experimental results were compared and their correlation was analysed with statistic methods.

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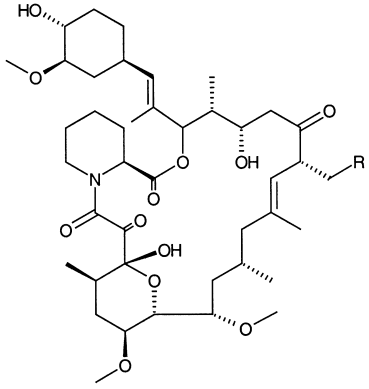
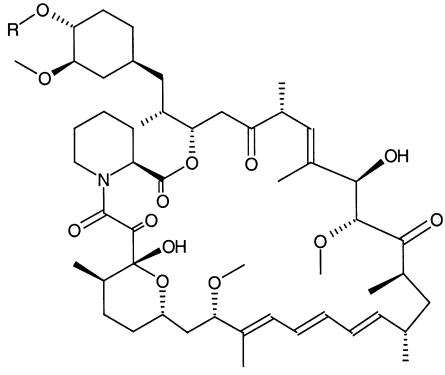


(**1**) R = $CH{=}CH_2$

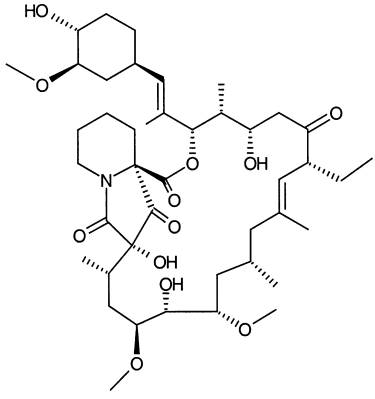
(**2**) R = Me

(**3**) R = H

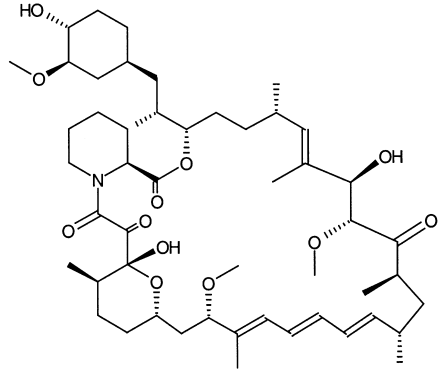


(**5**) R = H

(**6**) R = CH_2-CH_2OH



(**4**)



(**7**)

Figure 1. Structures of FK-506 (1), Ascomycin (2), Desmethylascomycin (3), DA-1 (4), Rapamycin (5), DR-1 (6) and DR-2 (7).

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Table 1

Macrolide Compounds used for Measuring Partition Coefficients

Nr.	Compound	MF	MW	Source	Ref
1	FK-506	$C_{44}H_{69}NO_{12}$	804	*Streptomyces tsukubaensis* No. 9993	16
2	Ascomycin	$C_{43}G_{69}NO_{12}$	792	*S. hygroscopicus* subsp. *yakushimaensis* No. 7238	17
3	Desmethylascomycin	$C_{42}H_{67}NO_{12}$	778	*S. hydroscopicus* subsp. *yakushimaensis* No. 7238	18
4	DA-1	$C_{43}H_{69}NO_{12}$	792	Synthetic derivative of ascomycin	19
5	Rapamycin	$C_{51}H_{79}NO_{13}$	914	*S. hydroscopicus*	20
6	DR-1	$C_{53}H_{83}NO_{14}$	958	Synthetic derivative of rapamycin	21
7	DR-2	$C_{51}H_{81}NO_{12}$	900	Synthetic derivative of rapamycin	22

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Determination of Partition Coefficients with the CCC Method

As shown in Equation 2, the calculation of partition coefficients in the CCC method is based on the retention volume of a given solute (V_r), the mobile phase volume in coils (V_m) and the total volume of the coils (V_t). The precision of calculated partition coefficients depends on, to great extent, the suitable proportions among V_r, V_m and V_t. It was reported that the retention volume of a given solute (V_r) must be at least 5 mL higher than the mobile phase volume in the coils (V_m) to determine partition coefficients with less than 10% of error.[24] Therefore, selection of mobile phase/stationary phase and elution mode in CCC is the first key step and plays the most important role.

In our experiment with hexane - tert-butylmethyl ether - methanol - water (1 : 3 : 6 : 5) , the organic layer was filled in coils as stationary phase while the aqueous phase was pumped through the column as mobile phase. The elution is in a "head to tail" model with "forward" direction. The difference of the retention volumes (V_r) of seven macrolides and the mobile phase volumes in the coils (V_m) is between 23 mL (5, rapamycin) and 462 mL (7, DR-2), much higher than the suggested 5 mL in literature.[24] This ensures our precise determination of partition coefficients with CCC. Detailed experimental data and results are summarised and presented in Table 2.

Determination of Partition Coefficients with the Shake-Flask Method

Although a number of experimental and theoretical models and methods have been developed to measure and estimate partition coefficients of com-

Table 2

Measurement of Partition Coefficients of Macrolide Compounds by CCC Method

Nr.	Compound	Rotation Speed (rpm)	Flow Rate (mL/min)	t_r (min)	V_t (mL)	V_m (mL)	V_r (mL)	$K_{(CCC)}$	log $K_{(CCC)}$
1	FK-506	800	3	94.2	300	95	282.6	0.915	-0.039
2	Ascomycin	800	3	87.5	300	95	262.5	0.817	-0.88
3	Desmethyl-ascomycin	700	3	76.0	300	87	228.0	0.662	-0.179
4	DA-1	800	3	39.5	300	95	118.5	0.115	-0.941
5	Rapamycin	700	3	126.0	300	87	378.0	1.366	0.136
6	DR-1	700	3	102.0	300	87	306.0	1.028	0.012
7	DR-2	700	3	183.0	300	87	549.0	2.169	0.336

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pounds,[25] the classical shake-flask method remains the standard method. In our experiments, the macrolides were partitioned between two immiscible phases of hexane - tert-butylmethyl ether - methanol - water (1 : 3 : 6 : 5), the same solvent system as used in CCC. The concentrations of analytes in both organic and aqueous phases were determined by means of HPLC. In comparison with the traditional spectrophotometric analysis in the shake-flask method, some of the disadvantages, for instance time consumption and various perturbing factors, can be avoided by HPLC analysis. Some limitations, such as the purity and stability of samples, as well as concentrations of solutions, can also be overcome. Moreover, the sensibility, reproducibility, and precision of the method, can be significantly improved by quantitative HPLC analysis. The partition coefficients of seven macrolide compounds measured with the shake-flask method are listed in Table 3, together with detailed experimental data.

Comparison of Partition Coefficients Determined by CCC and the Shake-Flask Method

The partition coefficients of seven macrolide compounds have been measured with both CCC and shake-flask method. The experimental results are presented in Table 2 and Table 3. The correlation between partition coefficients measured by CCC [log $K_{(CCC)}$] and by shake-flask method [log $K_{(SF)}$] was statistically analysed with Microcal™Origin™. A single linear relationship was found:

$$\log K_{(CCC)} = 0.980\ (\pm 0.017)\ \log K_{(SF)} - 0.008\ (\pm 0.007) \qquad (3)$$

$n = 7$, $r^2 = 0.998$, SD = 0.017, F = 3241

Table 3

Measurement of Partition Coefficients of Macrolide Compounds by Shake-Flask Method

Nr.	Compound	HPLC-UV (nm)	t_r (min)	$[A]_{org}$	$[A]_{aq}$	$K_{(SD)}$	Log $K_{(SF)}$
1	FK-506	210	17.5	50573	55125	0.917	-0.038
2	Ascomycin	210	15.8	49082	59641	0.823	-0.085
3	Desmethyl-ascomycin	210	12.2	46074	70125	0.657	-0.182
4	DA-1	210	16.8	1869	16265	0.115	-0.939
5	Rapamycin	275	33.7	5468	3903	1.401	0.146
6	DR-1	275	32.1	20146	20229	0.996	-0.002
7	DR-2	275	7.8	63410	26505	2.392	0.379

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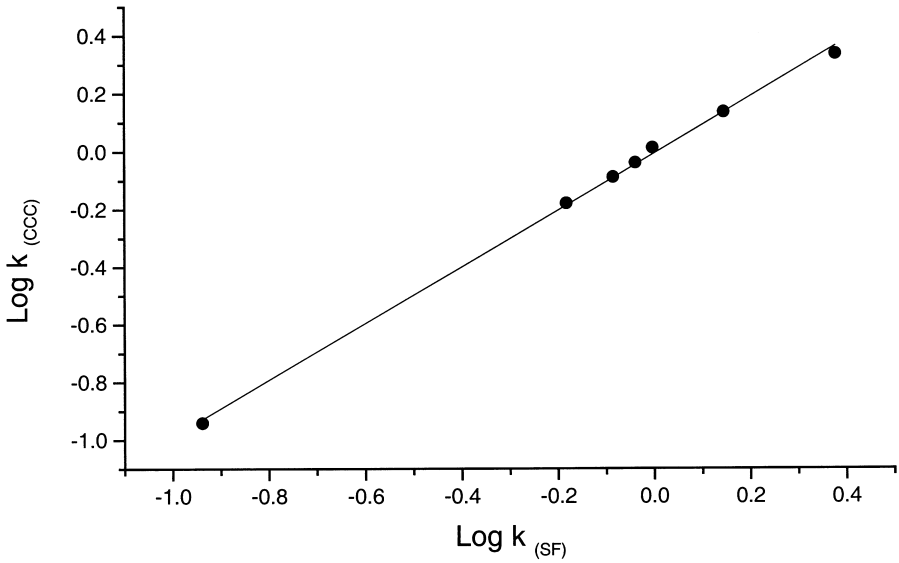

Figure 2. Relationship between log $K_{(CCC)}$ and log $K_{(SF)}$ for Seven Macrolide Compounds.

where n is the number of macrolide compounds, r^2 the square of the correlation coefficient, SD the standard deviation of the linear regression, and F is the Fischer test of the statistical significance of the equation. The values in parentheses are the 95% confidence limits of the regression coefficients. The Equations (3) and Figure 2 show that partition coefficient values measured by CCC [log $K_{(CCC)}$] are highly correlated with those determined by shake-flask method [log $K_{(SF)}$].

Correlation Between Partition Coefficient and Polarity

By comparing the log $K_{(CCC)}$ values of the seven macrolides, it is obvious that their partition coefficients were decreased as their polarity increased. As for three analogues of the FK-506 family (desmethylascomycin, ascomycin, and FK-506), their polarity agrees with that of the three substituent groups (R in Figure 1): H (desmethylascomycin) > CH_3 (ascomycin) > $CH{=}CH_2$ (FK-506). The decrease of their polarity results in the increase of their log $K_{(CCC)}$: -0.179 (desmethylascomycin) < -0.088 (ascomycin) < -0.039 (FK-506). With more hydroxyl groups in the molecule, the polarity of DA-1 is much higher than that of the other three compounds of FK-506 family. Therefore, the log $K_{(CCC)}$ value of DA-1 is also much lower (-0.941). Similarly, the three analogues in the rapamycin family show the same correlation between their polarity (DR-1> rapamycin >DR-2) and their partition coefficients (0.012 (DR-1) < 0.136 (rapamycin) < 0.336 (DR-2)).

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CONCLUSIONS

The partition coefficients of macrolide antibiotic analogues have been measured by CCC and compared with that determined by shake-flask method. The results indicate that the CCC method is a rapid and precise way for direct determination of log K. Partition coefficient values measured by CCC are highly correlated with those determined by shake-flask method. The advantages of the CCC method, such as rapidity, precision, and the ability to determine log K of analytes in a mixture, suggest that CCC can be a promising alternative to directly determine partition coefficients of macrolide antibiotics analogues in the purification process development.

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