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Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 871–878
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Relation between retention factors of immunosuppressive drugs in microemulsion electrokinetic chromatography with biosurfactants and octanol–water partition coefficients

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Received 13 May 2003; received in revised form 17 June 2003; accepted 20 June 2003

Abstract

Retention (capacity) factors (k' values) of immunosuppressive drugs were determined in microemulsion electrokinetic chromatography (MEEKC) systems as a tool for the indirect estimation of partition coefficients (P_{OW}) between 1-octanol and water. The microemulsions were based on phosphatidylcholine (PC) and bile acids (BAs) as biosurfactants and isopropyl myristate (IPM) as oil. Immunosuppressants were azathioprine (AZA), mycophenolate mofetil (MMF), tacrolimus (FK506) and cyclosporine A (CyA). Capacity factors of the analytes were determined from electrophoretic mobilities using an aqueous phosphate buffer (20 mM; pH 7.5) for all the systems. Retention was compared with that in the most commonly used microemulsion based on sodium dodecyl sulphate (SDS). $\log P_{OW}$ versus $\log k'$ calibration lines were constructed using reference compounds with known P_{OW}. In addition, data of $\log P_{OW}$ of the immunosuppressants were determined by partitioning between octanol and water, and were calculated by the aid of computer program. A different sequence of $\log P_{OW}$ for two analytes was found in the biosurfactant-based systems compared with the SDS-containing one. Excellent agreement was observed between the $\log P_{OW}$ values derived from the microemulsions containing deoxycholate compared with the data determined by partitioning between octanol and water. It was concluded that the retention factors in the systems with biosurfactants are good estimators for the partitioning in biological systems.

Keywords: Microemulsion electrokinetic chromatography; Immunosuppressive drugs; Lipophilicity estimation; Phosphatidylcholine; Bile acids

1. Introduction

Lipophilicity is considered as one of the most important physico-chemical properties in the design process of a new bioactive substance. Lipo-

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0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00377-7

philicity can be related to biological activities such as absorptivity, transportation, bioaccumulation, effectivity and toxicity, and is considered as a measure reflecting many biochemical and biophysical aspects of the interaction of drugs with biomembranes. One widely accepted reference scale of lipophilicity is based on the 1-octanol/water partition coefficient, P_{OW} (or its logarithm, log P_{OW}) which often shows good correlation with a number of pharmacological properties of drugs. Although 1-octanol/water partition coefficient values are usually determined following the standard shake-flask method [1,2], they are also estimated by the aid of liquid chromatography such as TLC [3,4] or HPLC [5–8], or by different mathematical models. More recently electrokinetic chromatography methods based on micelles [9–12], vesicles [13,14] or microdroplets (microemulsion electrokinetic chromatography, MEEKC) [15–19] have also turned out as reliable, rapid and economical alternatives for the indirect measurement of the hydrophobicity of compounds.

The analyte retention could serve as a measure to estimate the agreement between their octanol/water partition coefficients and their partitioning in the MEEKC systems. Common microemulsions consist of (i) an alkane like n-octane as lipophilic organic solvent, (ii) sodium dodecyl sulphate (SDS) as the additive that implements charges to the micro-droplets, and (iii) one of the lower alcohols as so-called co-surfactant (a more hydrophilic organic solvent), mostly in alkaline aqueous buffer (to establish cathodic electroosmotic flow (EOF) in fused silica capillaries). However, as the present work deals with bioactive analytes-several immunosuppressive drugs—we use microemulsions containing rather biosurfactants such as phosphatidylcholine (lecithine, PC) and bile salts (sodium cholate, SC and sodium deoxycholate, SDC) [20] than the common surfactants as pseudo-stationary phases, although the latter (based on SDS as surfactant) was implemented in the present work for comparison as well.

The inmunosuppressants selected were azathioprine (AZA) [21], mycophenolate mofetil (MMF) [22], tacrolimus (FK506) [23] and cyclosporine A (CyA) [24,25]. Their chemical structures are shown in Fig. 1. These therapeutic agents—frequently used to prevent rejection of transplanted organs—are characterised by their potent pharmacological activity, although with the disadvantage being connected with high adverse effects.

2. Experimental

2.1. Reagents and solutions

AZA, CyA, hydroquinone, resorcinol, benzyl alcohol, phenol, *m*-cresol, anisole, 2-naphthol, benzophenone, naphthalene, anthracene, dodecaphenone, SC, SDC, SDS, were purchased from Sigma (St. Louis, MO, USA). Epikuron 200 (PC, 95%) was a gift from Degussa Health and Nutrition (Freising, Germany), FK506 from Fujisawa Pharmaceutical Co. Ltd., Japan, and MMF from Roche Laboratory Argentina. Sodium monohydrogen phosphate, 85% phosphoric acid, n-octane, 1-butanol, isopropyl myristate (IPM), methanol and acetonitrile were HPLC grade and supplied by E. Merck (Darmstadt, Germany). Ultrapure water was obtained from EASY pure™ RF equipment (Barnstead, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations Inc., USA) and degassed before use.

Stock solutions containing 1.0 mg ml^{-1} of each drugs were prepared in methanol. Standard solutions of 40 μg ml^{-1} of AZA and MMF, 150 μg ml^{-1} of FK506 and 100 μg ml^{-1} for CyA were obtained by appropriate dilution with the microemulsion for the MEEKC (SDS) system. For MEEKC (PCSC/SDC) an appropriate aliquot of stock solution in methanol was evaporated and then re-dissolved in the microemulsion.

Stock and standard solutions of the reference compounds hydroquinone, resorcinol, benzyl alcohol, anisole, 2-naphtol, benzophenone, naphtalene and anthracene containing 1 mg ml^{-1} were prepared in methanol, those of phenol and *m*-cresol solutions (10 mg ml^{-1}) were prepared in water. Standard solutions containing 25 μg ml^{-1} anthracene, 200 μg ml^{-1} hydroquinone, resorcinol, anisole, 2-naphthol, benzophenone and naphthalene, 100 μg ml^{-1} benzyl alcohol and 1000 μg ml^{-1} phenol and *m*-cresol each were prepared by

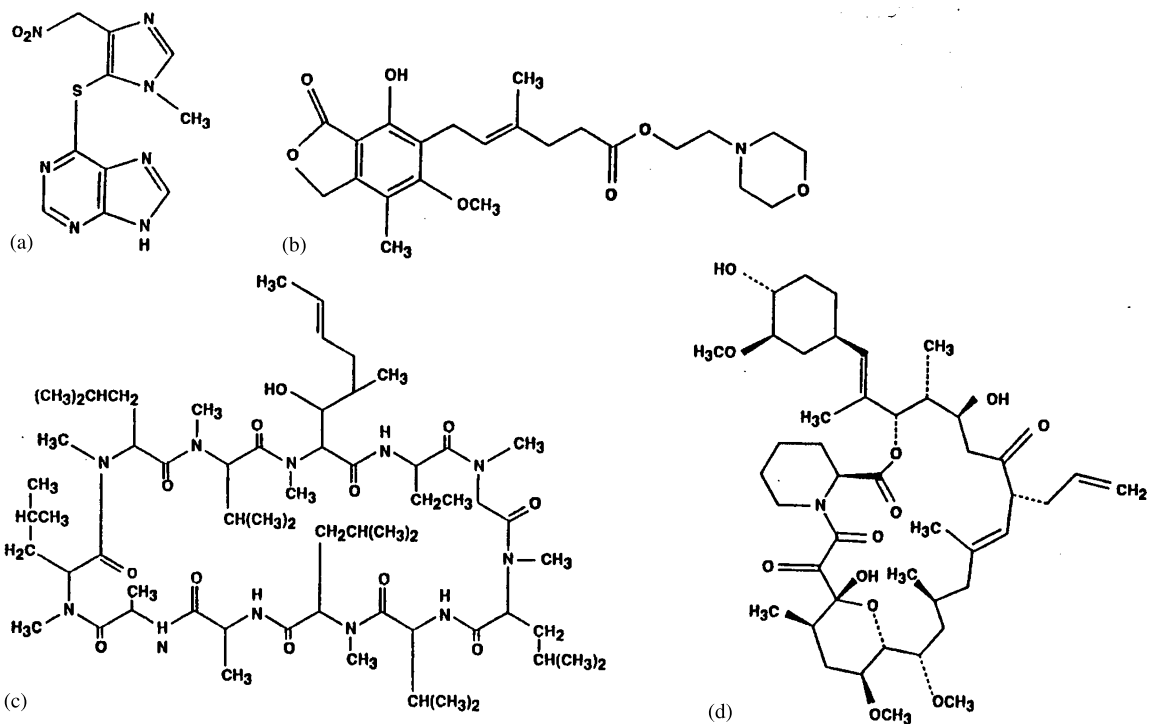

Fig. 1. Chemical structures of immunosupressive drugs (a) AZA, (b) MMF, (c) CyA and (d) FK506.

appropriate dilution with the corresponding microemulsion.

2.2. Instrumentation

Electrokinetic chromatography was performed with a Capillary Ion Analyser (Waters Corp., Milford, MA, USA); the data were processed by MILLENNIUM software (Waters). An uncoated fused-silica capillary of 60 cm length (53 cm to detector) and 75 μm I.D. (Waters) was employed. Hydrostatic injection (10 cm height) was for 15 s, voltages of 22 kV for MEEKC (SDS) and 25 kV for MEEKC (PCSC/SDC) were used. UV detection was at 214 nm (zinc lamp). The capillary was thermostated at 25 °C.

2.3. Microemulsion electrokinetic chromatography systems

The electrophoretic systems under study were: MEEKC (SDS), sodium phosphate (pH 7.5; 20 mM; 91.14%), octane (0.81%), SDS (1.44%) and 1-butanol (6.61%). MEEKC (PCSC), sodium phosphate (pH 7.5; 20 mM; 85.1%), IPM (1.9%), PC (3.5%), SC (2.0%) and 1-butanol (7.5%). MEEKC (PCSDC), sodium phosphate (pH 7.5; 20 mM; 85.1%), IPM (1.9%), PC (3.5%), SDC (2.0%) and 1-butanol (7.5%) [26]. Percentages are in w/w.

For preparation, the weighted amounts of surfactants, alcohol and oil were mixed by sonication during 5 min. Buffer was then slowly added and sonicated until a clear solution was obtained. The solution was let to stand for 30 min at room temperature before use.

2.4. Conditioning of the capillary

For the systems MEEKC (PCSC) and MEEKC (PCSDC) the capillary was rinsed at the beginning of each day with 0.1 M potassium hydroxide at 40 °C for 5 min, washed with water for 10 min at the same temperature and then with BGE for 20 min at 25 °C. Between runs, the capillary was

conditioned with the BGE for 3 min. At the end of the day, the capillary was flushed with 0.1 M potassium hydroxide during 5 min, and finally with water for 10 min at 40 °C. The capillary conditioning for the system MEEKC (SDS) was the same, except that it was carried out at 25 °C.

2.5. Experimental determination of partition coefficient P_{OW}

Partition coefficients between 1-octanol and phosphate buffer (pH 7.5; 20 mM) were determined according to the standard procedure described in [2]. The analyte concentration in the aqueous buffer phase was measured by MEEKC (SDS) following a procedure previously reported [27].

3. Results and discussion

In practice, a microemulsion is an optically clear system containing water, oil, surfactant and co-surfactant [28]. Systems based on PC and bile acids (BAs) like those under study were confirmed as microemulsions by phase diagrams whose zones were constructed by titrating phosphate buffer and oil with a solution of surfactant and co-surfactant [28,29], and by the size of microdroplets (5–7 nm) estimated by a simple approach as proposed by Kawakami et al. [30]. In order to mimic physiological acid–base conditions, all electrokinetic microemulsion systems used in the present work consist of aqueous buffer phosphate at pH 7.5.

Electrochromatograms of the immunosuppressants in the different electrophoretic systems under examination are shown in Fig. 2. While these two analytes cannot be resolved using MEEKC (PCSC), an improvement of selectivity is found for microdroplets of the MEEKC system composed of SDC or SDS instead of SC. Moreover, a reversal in the migration order of CyA and FK506

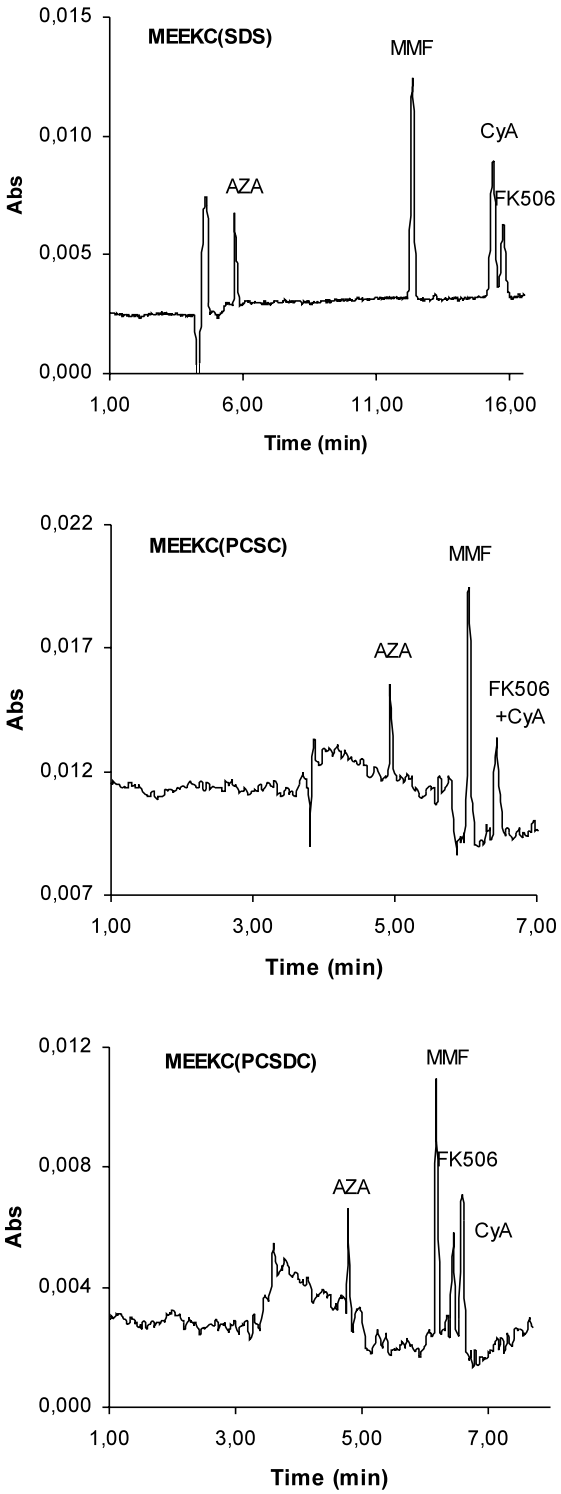

Fig. 2 (*Continued*)

Fig. 2. Chromatograms obtained by the different MEEKC systems. Composition of the systems are described in Section 2. Symbols of the analytes see Fig. 1.

Table 1
Electrophoretic mobilities and retention factors in the different MEEKC systems (mobilities are in 10^{-9} m^2 V^{-1} s^{-1})

Analyte	MEEKC (SDS)[a]			MEEKC (PCSC)[b]			MEEKC (PCSDC)[c]		
	$\mu_{i,\text{meas}}$	$\mu_{i,\text{eff}}$	k'	$\mu_{i,\text{meas}}$	$\mu_{i,\text{eff}}$	k'	$\mu_{i,\text{meas}}$	$\mu_{i,\text{eff}}$	k'
Aza	44.1	−13.9[a]	0.23	42.4	−13.1[b]	0.25	44.5	−14.8[c]	0.37
MMF	19.2	−38.7	8.02	35.0	−20.1	16.1	34.7	−22.6	9.71
FK506	14.9	−42.5	66.8	33.5	−20.6	28.3	32.9	−23.8	21.6
CyA	15.4	−42.1	39.4	33.0	−21.0	66.8	32.1	−24.3	39.7

$\mu_{\text{mic,eff}}$: [a] −43.2; [b] −21.3; [c] −24.9 (10^{-9} m^2 V^{-1} s^{-1}). $\mu_{i,\text{eff}}^{\text{free}}$ (AZA); [a] −7.2; [b] −11.1; [c] −11.1 (10^{-9} m^2 V^{-1} s^{-1}).

in MEEKC (SDS) compared with MEEKC (PCSDC) is observed.

3.1. Mobilities and retention (capacity) factors

From the electrochromatograms the mobilities of the analytes were determined as usual by their migration times. The mobilities of the EOF and those of the microdroplets were calculated using methanol and dodecaphenone, respectively, as markers in all electrophoretic systems. In the calculation of the own electrophoretic mobility of AZA in the cationic form, instead of the MEEKC (SDS) electrolyte one composed only of phosphate buffer (pH 7.5; 20 mM) and 6.6% 1-butanol was used; accordingly for the MEEKC (PCSC/SDC) system a phosphate buffer (pH 7.5; 20 mM) and 7.5% 1-butanol were employed.

The following precision of the migration time measurements expressed as relative standard deviations (RSD values) were obtained. Intra-day precision was 0.6–1.0% for the analytes (average from six runs each); 0.7–1.3% for the EOF, 1.0 and 1.1% for the microdroplets. RSD for inter-day (average from 18 runs at 3 different days) for analytes was 0.8–1.3, 1.0–1.5% for the EOF, and 1.1–1.4% for the microdroplets. A remarkably high inter-day reproducibility of the microemulsion systems could be evidenced in comparison to capillary zone electrophoresis.

Table 1 presents the values of electrophoretic mobilities of the drugs in the different MEEKC systems. It can be seen that the measured mobilities, which depend on the EOF in the particular systems, range between 15 and 45 units (mobility units are given here as (10^{-9} m^2 V^{-1} s^{-1})). It is obvious that they have negative sign, as they are transported in the micelles against the EOF. The effective mobilities (those corrected by the EOF mobility) have the widest range in the SDS-system, and are in a similar span in the PCSC-and the PCSDC-systems. However, the mobilities do not give a clear picture about the extent of partitioning between the aqueous phase and the microdroplet. This is better expressible by the retention or capacity factor, k'.

k' is defined as the mass distribution coefficient according to:

$$k' = \frac{n_{i,\text{s}}}{n_{i,\text{m}}} = \frac{c_{i,\text{s}} V_\text{s}}{c_{i,\text{m}} V_\text{m}} \tag{1}$$

where $n_{i,\text{s}}$ and $n_{i,\text{m}}$ are the mole numbers of analyte, i, in the pseudo-stationary (s) and mobile phase (m), respectively. c_i are the molar concentrations of the analyte in the respective phases with volumes V_s and V_m. The values of the retention factors can be calculated from the mobilities according to the following equation [31–33]:

$$k' = \frac{\mu_{i,\text{meas}} - \mu_i^{\text{free}}}{\mu_{\text{mic}} - \mu_{i,\text{meas}}} \tag{2}$$

Here $\mu_{i,\text{meas}}$ is the measured mobility of the analyte i, in the electrochromatographic system, μ_i^{free} is its mobility in free solution; μ_{mic} is the mobility of the microdroplet. The effective mobilities are the measured mobilities corrected by that of the EOF. The ionic mobility of a charged analyte (as it is AZA) in free solution has to be known for the correct calculation of k' values.

The corresponding retention factors calculated from the mobilities by Eq. (2) are given in Table 2 also. AZA has the lowest k' value; this cation is

Table 2
log k' values of the reference compounds

Compounds		MEEKC (SDS)	MEEKC (PCSC)	MEEKC (PCSDC)
	log P_{OW}[a]	log k'	log k'	log k'
Hydroquinone	0.55	−0.780	−0.609	−0.257
Resorcinol	0.80	−0.591	−0.078	−0.106
Benzyl alcohol	1.10	−0.326	−0.009	−0.003
Phenol	1.46	−0.221	0.317	0.284
m-Cresol	1.96	0.103	0.637	0.635
Anisole	2.11	0.306	0.878	0.825
β-Naphthol	2.84	0.586	1.180	0.888
Benzophenone	3.18	0.932	1.149	0.919
Naphtalene	3.37	1.043	1.259	1.210
Anthracene	4.45	1.524	2.046	1.720

[a] Data of log P_{OW} are taken from [10,15–17,19].

partitioning into the microdroplet only to a small extend. The other analytes exhibit largest k' values of more than 60 in the SDS and PCSC systems. It is noticeable that the sequence of the k' values is not the same in all systems.

3.2. Retention factor and 1-octanol/water partition coefficient

The partition coefficients, P, of the analytes between 1-octanol and water can be determined from their k' values using reference compounds whose P_{OW} values are known. Note that we indicate as P_{OW} the partition coefficient that is explicitly determined by distribution between octanol and water, and as P that indirectly derived from k'. For the present work we use the reference compounds listed in Table 2, with log P_{OW} data (taken from the literature) ranging between 0.55 and 4.45. These data allow construction of a calibration line for each microemulsion electrophoretic system with the measured log k' values, presented in Table 2, according to:

$$\log P_{OW} = a \log k' + b \quad (3)$$

where a and b are the regression constant and the regression coefficient, respectively. The following results were obtained. MEEKC (SDS): log P_{OW} = 1.6 log k' + 1.7 ($r = 0.9958$; s(slope) 0.05, s(intercept) 0.04); MEEKC (PCSC): log P_{OW} = 1.6 log k' + 1.1 ($r = 0.9802$; s(slope) 0.11, s(intercept) 0.11); MEEKC (PCSDC): log P_{OW} =

Table 3
log P of immunosuppressive drugs determined from log k'

Analyte	log P_{OW}		log P		
	Software program	Experimental	MEEKC (SDS)	MEEKC (PCSC)	MEEKC (PCSDC)
Aza	−0.54[a]	0.10[c] 0.15[d]	0.68	0.18	0.12
MMF	4.01[a]	3.18[d]	3.15	3.01	2.98
FK506	3.96[a]	3.77[d]	4.62	3.40	3.67
CyA	14.00[b]	4.30[d]	4.25	3.99	4.20

[a] From Software Solaris [35].
[b] From [34].
[c] From [36].
[d] From this study.

2.0 log k' + 1.01 ($r = 0.9817$; s(slope) 0.13, s(intercept) 0.11); s is the standard deviation.

The (indirectly determined) coefficient P can be derived from the k' values of the analytes upon calibration with the measured k' values of the reference compounds. log P of the analytes is then:

$$\log P = a \log k' + b \quad (4)$$

with the values for a and b derived from Eq. (3) and given above. The log P values of the immunosuppressive drugs obtained in this way are shown in Table 3.

It can be seen that the partition coefficients cover nearly four orders of magnitude, from a coefficient around 1 (for AZA in PCSDC) to around 40.000 (for FK506 in SDS). The two PC-containing systems show the same sequence: log P values increase in the order AZA < MMF < FK506 < CyA. However, the order reverses for FK506 and CyA in the SDS-system, indicating a difference in selectivity compared with the two others. It is, therefore, valuable to inspect which sequence correlates with the corresponding P_{OW} data.

3.3. Relation between log P_{OW} from octanol/water partitioning and log P from MEEKC

log P_{OW} data were, therefore, experimentally determined from partitioning between octanol and water as usual. They are given in Table 3 together with those calculated by the aid of a software program. It may be mentioned that the experimentally determined log P_{OW} values of CyA significantly differ from that calculated by the algorithm [34], showing the limitations of a computational approach when complex structures are considered. Indeed CyA, a cyclic undecapeptide, presents different partitioning behaviour depending on the polarity and hydrogen donor capacity of the solvent used [34].

Evaluation of the relationship between octanol/water partition and MEEKC partition by plotting log P_{OW} versus log P derived from the different electrokinetic systems is carried out by linear correlation; the following regression coefficients are obtained: 0.9714 for MEEKC (SDS), 0.9993 for MEEKC (PCSC) and 0.9994 for MEEKC (PCSDC). It can be seen that MEEKC systems with the biosurfactants PC, BA and IPM are higher correlated to octanol/water than that consisting of SDS.

4. Conclusions

Microemulsion chromatography was used as a robust method for the determination of log P values from log k' with high intra- and inter-day precision. Comparison of log P in the MEEKC system with the experimentally determined log P_{OW} data (from octanol/water partitioning) show an excellent agreement of the latter with the deoxycholate containing system. The data for all analytes correspond within few percent. PCSC gives a slightly lower agreement, but the sequence of log P and log P_{OW} values is the same. In contrast, in the SDS-containing microemulsion system the order reverses for FK506 and CyA, indicating that chromatographic retention poorly reflects octanol–water partitioning here. It follows that log P values based on lecithin MEEKC systems seems to be better descriptors for estimation of the lipophilicity of immunosuppressive drugs. In the pharmaceutical technology field they might also be applied as better models of drug releasing in the development of microemulsion based formulations.

Acknowledgements

The authors acknowledge providing drugs and reagents from Degussa Bioactives Germany and Degussa Argentina, Fujisawa Pharmaceutical, Roche Laboratory Argentina and Glaxo Welcome Argentina.

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