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Effects of ions and detergents in drug partition chromatography on liposomes

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Abstract

We have determined drug partitioning into phospholipid bilayers by immobilized-liposome chromatography (ILC). Electrostatic effects on the drug partitioning were observed on neutral bilayers at low ionic strength. The size of the counterions affected the partitioning. When liposomes were supplemented with ionic detergents the partitioning of charged drugs was strongly affected, allowing complete separation of drugs of different charges which showed similar retention on neutral bilayers. Partial separation was obtained on bilayers containing fatty acid. Detergent ions or fatty acid inserted into phospholipid bilayers affected the partitioning of drugs much more than did free ions or phospholipid head group charges.

Keywords: Liposomes; Drug partitioning; Immobilized-liposome chromatography; Electrostatic effects; Ionic strength; Phospholipid bilayers; Surfactants; Drugs

1. Introduction

The diffusion of a solute across lipid bilayers involves the partitioning of the solute between the aqueous phases and the outskirts of the bilayers [1]. In drug screening, octanol-water-partitioning coefficients, P_{oct}, are used for estimating drug partitioning into membranes. P_{oct} reflects hydrophobic interactions, but not the additional electrostatic interactions between the drug molecules and the phospholipid head groups in the membrane. Electrostatic interactions affect the permeation of solutes across Caco-2 cells [2] and are important for drug delivery by use of liposomes, for example, oligonucleotide delivery in positive liposomes [3].

Liposomes resemble the natural membrane and methods that have been developed for studying the partitioning of drugs and other solutes into membranes or phospholipid layers are, for example, optical analysis [4–6], pH titration [7,8], immobilized artificial membrane chromatography [9–11], immobilized-liposome chromatography (ILC) [12–18] and biopartitioning micellar chromatography with $(ethyleneglycol)_{23}$ monododecyl ether [19,20]. These methods determine the partitioning into the entire membrane, although the partitioning into the hydrophobic region adjacent to the phospholipid head groups may be more relevant to drug permeation.

In the present work, we analyzed, by use of ILC, the effects of free ions, membrane-inserted detergent ions and a fatty acid on the partitioning of negative-, positive- and neutral-drugs into phospholipid bilayers. We also studied the effect of detergent ions or a fatty acid in the bilayers on the separation of charged and neutral drugs. The use of amphiphilic ions in ILC may facilitate analysis and purification of synthesized drugs and solve problems in screening of plant extracts for physiologically active compounds. Other modifications of ILC have recently been introduced for analysis of plant extracts [21–23].

2. Experimental

2.1. Materials and solutions

We purchased glass columns (HR 5, i.d. 5 mm) and Superdex 200 prep grade gel beads from Amersham Biosciences (Uppsala, Sweden) and 1-palmitoyl-2-oleoyl-*sn*-

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Table 1
Detergents used: abbreviation, formula and critical micellar concentration (CMC)

Detergent	Abbreviation	Formula	CMC
Octaethyleneglycol monododecyl ether	$C_{12}E_8$	$CH_3(CH_2)_{11}O(CH_2CH_2O)_8H$	60–90 μM[a]
Sodium dodecylsulfate	SDS	$CH_3(CH_2)_{11}OSO_3^-Na^+$	1.8 mM[b]
Dodecyltrimethyl-ammonium bromide	DTAB	$CH_3(CH_2)_{11}N^+(CH_3)_3Br^-$	5 mM[c]
Tetradecyltrimethyl-ammonium bromide	MTAB	$CH_3(CH_2)_{13}N^+(CH_3)_3Br^-$	4.5 mM[d]
Hexadecyltrimethyl-ammonium bromide	CTAB	$CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$	10 μM < CMC < 50 μM[e]

[a] Values from [24,25].
[b] Ionic strength (I) 0.1, value from [26].
[c] $I = 0.15$, value from [27].
[d] In water, value from [28].
[e] $I = 0.15$, our estimate on the basis of the stability of entrapped liposomes.

glycero-3-phosphocholine (POPC, >99%) from Avanti Polar Lipids (Alabaster, AL, USA). We bought the detergents listed in Table 1 from three sources: $C_{12}E_8$ (>98%) from Fluka (Buchs, Switzerland), SDS (>99%) from Merck (Darmstadt, Germany) and DTAB, MTAB and CTAB (all approx. 99%) from Sigma (St. Louis, MO, USA). We purchased arachidic acid (>99%), alprenolol, corticosterone, dexamethasone, hydrocortisone, pindolol and propranolol from Sigma. Bupivacaine, desmethyldiazepam, gemfibrozil, ibuprofen, indomethacin, indoprofen, lidocaine, metolazone, metoprolol, naproxen, oxazepam, phenytoin, salicylic acid, sulpirid, theophylline, tolmetin and warfarin were gifts from Kirsti Gjellan (AstraZeneca, Södertälje, Sweden) and Paul Smith (SmithKline Beecham, King of Prussia, PA, USA).

Egg yolk phospholipids (EPLs) were prepared from hen's eggs as described in [29] to consist of phosphatidylcholine (70%), phosphatidylethanolamine (21%), other phospholipids and lysophospholipids (9%), and small amounts of cholesterol and other components [30].

Solution A consisted of 10 mM Tris and 150 mM NaCl, solution B of 1 mM Tris, solutions C–G of 5 mM Tris and 0, 50, 100, 150 or 200 mM NaCl, respectively, solutions H and I of 5 mM Tris and 50 or 150 mM LiCl, respectively, and solution J of 5 mM Tris and 50 mM LiF, all supplemented with 1 mM Na_2EDTA and adjusted with HCl to pH 7.4 at 21 ± 2 °C.

2.2. Immobilized-liposome chromatography

We entrapped liposomes in gel beads as described for (proteo)liposomes in [16]. A detailed description is given in [31]. In brief, we rehydrated a lipid film to form multilamellar liposomes, mixed the suspension with dried Superdex 200 prep grade beads, immobilized the liposomes by five cycles of freezing and thawing, washed the gel by centrifugation, packed it into a HR glass column and equilibrated with the eluent. We applied a 20 μl drug sample (0.1 mg/ml in the eluent, no detergent, <5% ethanol) at a flow rate of 0.5 or 1.0 ml/min at 21 ± 2 °C and monitored the elution at 220 nm. We made two or three runs for each determination unless otherwise stated.

We expressed the drug partitioning as the K_s value (M^{-1}), i.e., the retention of the drug per amount of phospholipid, A, in the gel bed. The A value was determined by phosphorus analysis [32] after the series of chromatographic runs. The equation below was used when calculating the K_s value:

$$K_s = \frac{V_E - V_0 - V_G}{A} \quad (1)$$

with V_E, the elution volume of the drug, V_0, the elution volume of a compound ($Cr_2O_7^{2-}$) that does not interact with the liposomes or the gel, and V_G, the retention volume of the drug on a liposome-free bed of the same type and size as the gel bed containing liposomes [16].

In experiments with detergent inserted into the bilayers, we equilibrated the entrapped liposomes with detergent-supplemented buffer. The detergent thereby partitioned into the bilayers to attain a density governed by the partition coefficient and the detergent concentration in the aqueous phase.

3. Results

3.1. Effect of ionic strength and size of ions on drug partitioning into liposomes

The drug partitioning into bilayers, as determined by ILC, showed electrostatic effects at low ionic strength (Fig. 1). The partitioning of negative drugs into the neutral POPC bilayers decreased gradually when the ionic strength was increased, whereas the partitioning of positive drugs increased strongly at extremely low ionic strength (I = 0.0003) (Fig. 1A). The EPL bilayers showed moderately increased partitioning of negative drugs and decreased partitioning of positive drugs when the ionic strength was increased from 0.003 to 0.05 (Fig. 1B). The presence of phosphatidylethanolamine gives the EPL bilayer a small negative net charge, which may contribute to the differences between POPC and EPL. The neutral drugs were essentially unaffected by the ionic strength in the two types of bilayers (Fig. 1). The effects of small ions, Li^+ versus Na^+, and F^- versus Cl^-, were tested on POPC liposomes

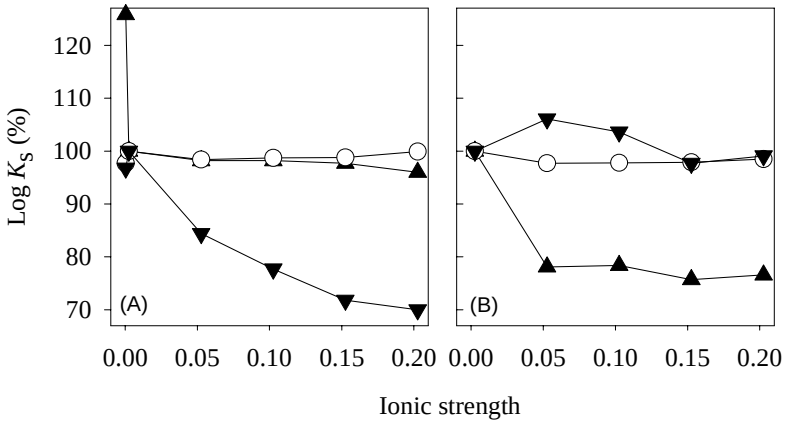

Fig. 1. Effects of ionic strength on the drug partitioning into phospholipid bilayers determined by ILC. Average $\log K_s$ values, expressed in percent, for negative (▼), positive (▲) and neutral (○) drugs are plotted versus ionic strength on (A) POPC liposomes (solutions B–G) and (B) EPL liposomes (solution C–G). The values at $I = 0.003$ are set to 100%. In (A), the points at 97, 126, and 98% are determined at $I = 0.0003$. The drugs were ibuprofen, indoprofen, naproxen and tolmetin (negative); bupivacaine, lidocaine, metoprolol, pindolol and propranolol (positive); and desmethyldiazepam, dexamethasone, metolazone, oxazepam and hydrocortisone (neutral). The standard errors were approx. ±1% ($n = 2$) in (A) and were estimated to be ±2% ($n = 1$) in (B).

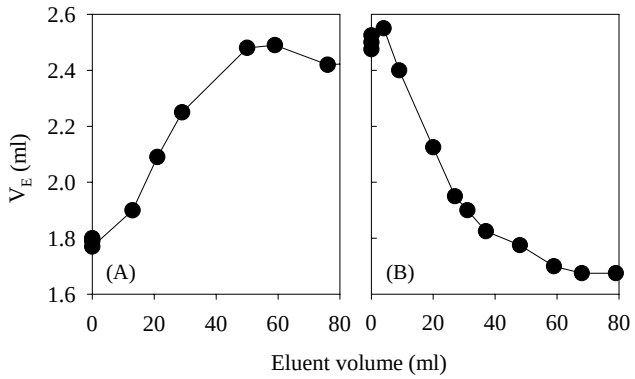

Fig. 2. The elution volume, V_E, of naproxen on EPL bilayers versus the eluent volume during (A) equilibration with DTAB (50 μM) in solution A, and (B) washing with solution A.

with the drugs used in Fig. 1 with solutions F and H–J as eluents (not illustrated). The average $\log K_s$ values for the negative drugs were 15% higher with LiCl than with NaCl at $I = 0.15$, whereas the partitioning of the positive drugs increased only slightly. The neutral drugs were essentially unaffected. The average $\log K_s$ values for the positive drugs were 8% higher with LiF than with LiCl at $I = 0.05$. The negative and neutral drugs were not affected.

3.2. Effects of detergents on drug partitioning into liposomes

We equilibrated entrapped liposomes with detergent in solution A. Runs of naproxen or propranolol monitored the equilibration and subsequent washing-out of the

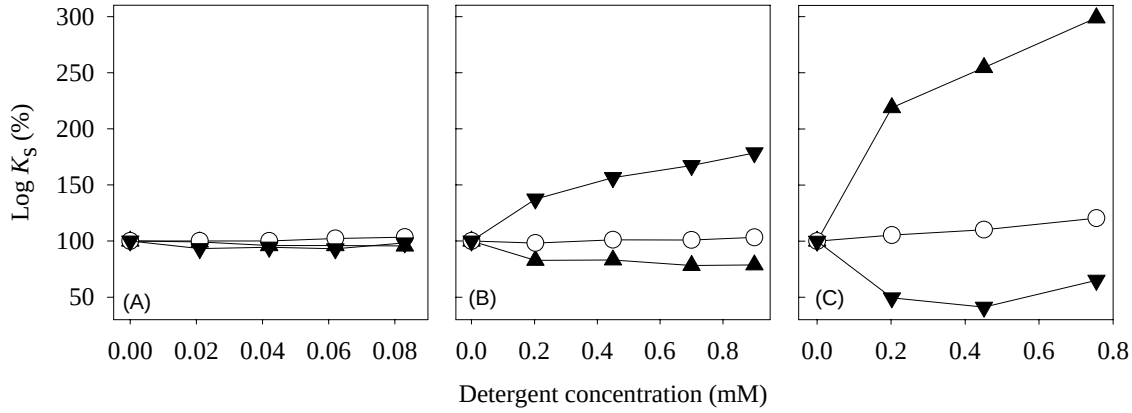

Fig. 3. Effects of charged detergents on drug partitioning into EPL bilayers determined by ILC with detergent-supplemented solution A. Average $\log K_s$ values, expressed in percent, for negative (▼), positive (▲) and neutral (○) drugs are plotted versus the concentration of (A) $C_{12}E_8$, (B) DTAB, and (C) SDS in solution A. The values at detergent concentration 0 are set to 100%. The drugs were indomethacin, naproxen and warfarin (negative), lidocaine and propranolol (positive), and oxazepam and phenytoin (neutral). Indomethacin and propranolol did not elute in (B) and (C), respectively. The standard errors were approx. ±1% ($n = 2$–3).

Table 2
Effects of increased ionic strength on drug partitioning into EPL bilayers equilibrated with 0.45 mM DTAB or SDS

	$\Delta \log K_s$[a]	
	DTA^+	DS^-
Negative drugs (naproxen, warfarin)	0.37	−0.21
Positive drug (lidocaine)	−0.13	0.12
Neutral drugs (oxazepam, phenytoin)	−0.08	−0.38

[a] The difference between the $\log K_s$ values determined at $I = 0.003$ (solution C) and $I = 0.15$ (solution A). The standard errors were less than ±0.06.

detergent from the bilayers. Each process required about 1 h at 1 ml/min for DTAB (Fig. 2), 3 h for MTAB, 12 h for CTAB, and 1 h for $C_{12}E_8$.

$C_{12}E_8$ affected the drug partitioning into EPL bilayers only slightly (Fig. 3A). The partitioning of negative drugs into bilayers supplemented with DTAB increased with increasing detergent concentration, that of positive drugs decreased moderately and neutral drugs were unaffected (Fig. 3B).

The partitioning of the negative drugs decreased with increasing concentration of SDS up to 0.5 mM, whereas the partitioning increased strongly for the positive drugs and moderately for the neutral drugs (Fig. 3C). The effects of the detergent ions were moderately enhanced at low ionic strength (Table 2).

3.3. Separation of drugs on immobilized liposomes equilibrated with detergent

Drugs of different charges but similar K_s values co-eluted as expected on the essentially neutral EPL liposomes (Figs. 4A and 5A). Upon adding detergent ions the net charge of the EPL bilayer was changed, which affected the retention volumes of the drugs and made it possible to separate drugs of opposite charges (Fig. 4B and C). The negative drug naproxen showed decreased elution volume in the presence of the negative detergent SDS (Fig. 4B), whereas the elution volume of the positive drug pindolol was increased. The opposite was shown with the positive detergent DTAB (Fig. 4C).

The partitioning of charged drugs run in MTAB- or CTAB-supplemented solution A was compared to the partitioning in bilayers equilibrated with DTAB. A negative, a positive and a neutral drug, which co-eluted in plain solution A, were separated, as exemplified in Fig. 5B–D. The retention volumes of negative drugs increased in the presence of MTAB and CTAB compared to DTAB, whereas the retention volumes decreased for positive drugs (Fig. 5B–D). The K_s values changed linearly with the length of the alkyl chain of the alkyltrimethylammonium ions (not shown). For the neutral drug corticosterone (ii) the $\log K_s$ values differed only slightly (2.43, 2.45, 2.60 and 2.80 in Fig. 5A–D, respectively).

3.4. Separation of drugs on immobilized liposomes prepared from phospholipid and fatty acid

We mixed the fatty acid arachidic acid with EPL to 5 or 50 mol% before the preparation of liposomes, immobilization and ILC. The elution volumes remained constant for all drugs during 4 days of running over a period of 11 days. Partial separation was achieved when we ran a mix of alprenolol, corticosterone and gemfibrozil (compare with EPL alone, Fig. 4A), since the retention volume was decreased for the negative drug. When we used 50 mol% arachidic acid the separation improved compared to 5 mol% of the fatty acid. However, only two peaks were obtained and baseline separation was not achieved in either case (data not shown).

4. Discussion

The enhanced retardation of negative drugs on neutral POPC bilayers at low ionic strength (Fig. 1A) indicates that, in this case, the drugs sensed the positive charges of the choline groups at the membrane surface. The positive drugs were less affected by the ionic strength, except at

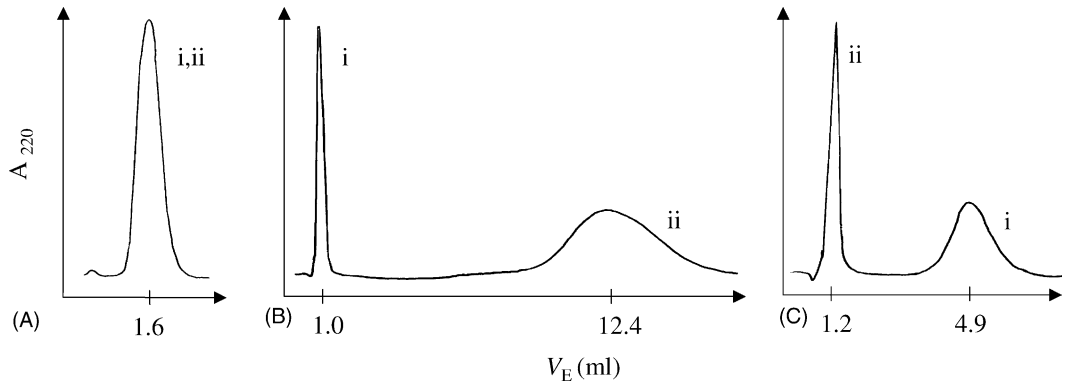

Fig. 4. ILC of naproxen (i, negative) and pindolol (ii, positive) on EPL bilayers in (A) solution A, and ILC separation of the drugs in solution A supplemented with (B) SDS (0.2 mM) and (C) DTAB (0.2 mM). The phospholipid amounts were 21 μmol in (A), 21 μmol in (B), and 22 μmol in (C), respectively.

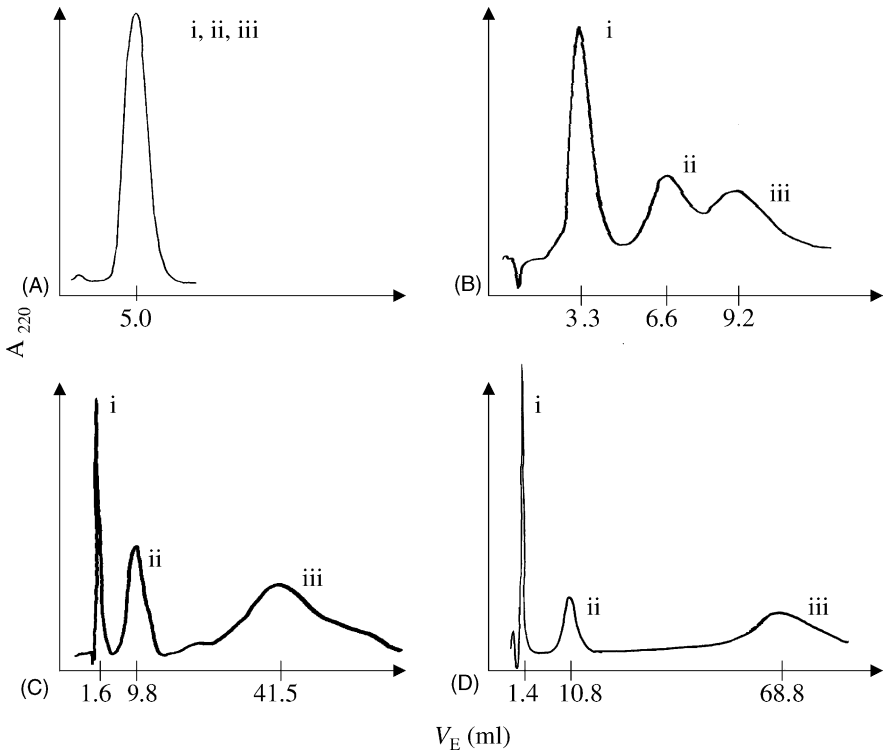

Fig. 5. ILC of alprenolol (i, positive), corticosterone (ii, neutral) and gemfibrozil (iii, negative) on EPL bilayers in (A) solution A, and ILC separation of the drugs in solution A supplemented with (B) DTAB (50 μM), (C) MTAB (50 μM) and (D) CTAB (10 μM). The phospholipid amounts were 33 μmol in (A), 45 μmol in (B), 33 μmol in (C), and 25 μmol in (D), respectively.

$I = 0.0003$, presumably since they interacted with the phosphate groups within the membrane. Small counter ions (Li^+, F^-) in the eluent modified the drug-bilayer interactions, perhaps because the smaller ions penetrated deeper into the head group region.

The drug interaction with phospholipid bilayers could be modulated by the presence of bilayer-inserted detergent in equilibrium with detergent in the eluent. The supplementation of detergent to the bilayers required an hour or more (Fig. 2A) because of the low detergent concentration in the eluent and the moderate flow rate. The detergent inserts rapidly into phospholipid bilayers according to [33,34], although slow flip-flop has been suggested previously [35,36]. After the equilibration the entrapped liposomes were stable, provided that the detergent concentration was kept below the critical micellar concentration (CMC) (Table 1). The bilayer/water partition coefficient of the detergent determines the resulting density of detergent molecules or ions in the lipid bilayers [25,33,34,36–38], which must not become too high. In the case of $C_{12}E_8$ the critical value corresponds to about 0.5–1 detergent molecule per phospholipid molecule [25,33,35,37]. Although nonionic detergents increase the permeability of phospholipid bilayers toward small solutes [39], the overall effect of $C_{12}E_8$ on the partitioning was small (Fig. 3A). The flexible hydrophobic chains of the nonionic detergent thus have smaller effects than does the rigid sterol skeleton of cholesterol, which decreases drug partitioning above the transition temperature of the bilayers [16].

Since the elution volume of charged drugs was changed considerably in the presence of detergent ions, oppositely charged drugs could be separated from each other and from neutral drugs (Figs. 4 and 5). Presumably the charged drugs interacted electrostatically with the detergent ion in addition to their hydrophobic interaction with the fatty acyl chain, which affected the partitioning considerably. The separation improved with the length of the carbon chain of the detergent (Fig. 5B–D) due to increasing partition coefficients and, hence, the concentration of the detergent ions in the bilayers.

The $\log K_s$ values for negative drugs seemed to partition more weakly into bilayers with arachidic acid, whereas positive drugs were unaffected. Probably the low permittivity in the lipid bilayer favored the neutral protonated form of the arachidic acid, which explains why the detergent ions showed stronger effects. Recently, we suggested that negative charges in intestinal brush border membranes increased the partitioning of positive drugs into the membrane [40] because the $\log K_s$ values for positive drugs decreased over time, perhaps due to loss of negative charges from the membrane. This effect differs from the effect of arachidic acid in our ILC analyses.

5. Conclusions

The free ions in the eluent or the zwitterionic phospholipid head groups affected the partitioning of the drugs less than

did detergent ions and a fatty acid. The strong effects of detergent ions on the partitioning of charged drugs into lipid bilayers can be useful for analytical or preparative separation of drugs.

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References

[1] W.D. Stein, Channels, Carriers, and Pumps—An Introduction to Membrane Transport, Academic Press, San Diego, CA, 1990, p. 29.
[2] M. Sugawara, M. Kurosawa, K. Sakai, M. Kobayashi, K. Iseki, K. Miyazaki, Biochim. Biophys. Acta 1564 (2002) 149.
[3] F. Shi, L. Wasungu, A. Nomden, M.C.A. Stuart, E. Polushkin, J.B.F.N. Engberts, D. Hoekstra, Biochem. J. 366 (2002) 333.
[4] J.J. Ramsden, Experientia 49 (1993) 688.
[5] E. Danelian, A. Karlén, R. Karlsson, S. Winiwarter, A. Hansson, S. Löfås, H. Lennernäs, M.D. Hämäläinen, J. Med. Chem. 43 (2000) 2083.
[6] C.L. Baird, E.S. Courtenay, D.G. Myszka, Anal. Biochem. 310 (2002) 93.
[7] A. Avdeef, K.J. Box, J.E.A. Comer, C. Hibbert, K.Y. Tam, Pharm. Res. 15 (1998) 209.
[8] K. Balon, B.U. Riebesehl, B.W. Müller, J. Pharm. Sci. 88 (1999) 802.
[9] S. Ong, H. Liu, C. Pidgeon, J. Chromatogr. A 728 (1996) 113.
[10] B.H. Stewart, O.H. Chan, J. Pharm. Sci. 87 (1998) 1471.
[11] A. Taillardat-Bertschinger, C.A. Marca Martinet, P.-A. Carrupt, M. Reist, G. Caron, R. Fruttero, B. Testa, Pharm. Res. 19 (2002) 729.
[12] E. Brekkan, L. Lu, P. Lundahl, J. Chromatogr. A 711 (1995) 33.
[13] F. Beigi, I. Gottschalk, C. Lagerquist Hägglund, L. Haneskog, E. Brekkan, Y. Zhang, T. Österberg, P. Lundahl, Int. J. Pharm. 164 (1998) 129.
[14] Q. Yang, X.-Y. Liu, M. Yoshimoto, R. Kuboi, J. Miyake, Anal. Biochem. 268 (1999) 354.
[15] Q. Yang, X.-Y. Liu, K. Umetani, N. Kamo, J. Miyake, Biochim. Biophys. Acta 1417 (1999) 122.
[16] C. Lagerquist, F. Beigi, A. Karlén, H. Lennernäs, P. Lundahl, J. Pharm. Pharmacol. 53 (2001) 1477.
[17] T. Österberg, M. Svensson, P. Lundahl, Eur. J. Pharm. Sci. 12 (2001) 427.
[18] X.-Y. Liu, C. Nakamura, Q. Yang, N. Kamo, J. Miyake, J. Chromatogr. A 961 (2002) 113.
[19] M. Molero-Monfort, Y. Martín-Biosca, S. Sagrado, R.M. Villanueva-Camañas, M.J. Medina-Hernández, J. Chromatogr. A 870 (2000) 1.
[20] C. Quiñones-Torrelo, Y. Martín-Biosca, J.J. Martínez-Pla, S. Sagrado, R.M. Villanueva-Camañas, M.J. Medina-Hernández, Mini Rev. Med. Chem. 2 (2002) 145.
[21] X. Mao, L. Kong, H. Wang, H. Zou, Chin. J. Anal. Chem. 30 (2002) 231.
[22] X. Mao, L. Kong, Q. Luo, X. Li, H. Zou, J. Chromatogr. B 779 (2002) 331.
[23] X. Mao, L. Kong, X. Li, B. Guo, H. Zou, Anal. Bioanal. Chem. 375 (2003) 550.
[24] M.N. Jones, Int. J. Pharm. 177 (1999) 137.
[25] H. Heerklotz, J. Seelig, Biophys. J. 78 (2000) 2435.
[26] K. Ibel, R.P. May, K. Kirschner, H. Szadkowski, E. Mascher, P. Lundahl, Eur. J. Biochem. 190 (1990) 311.
[27] C. Tanford, The Hydrophobic Effect: Formation of Micelles and Biological Membranes, second ed., Wiley, New York, NY, 1980, p. 60.
[28] J.B.C. Findlay, in: E. Harris, S. Angal (Eds.), Protein Purification Applications: A Practical Approach, IRL Press, New York, NY, 1990, p. 59.
[29] E. Mascher, P. Lundahl, Biochim. Biophys. Acta 945 (1988) 350.
[30] Q. Yang, P. Lundahl, Anal. Biochem. 218 (1994) 210.
[31] E. Brekkan, Q. Yang, G. Viel, P. Lundahl, in: G.F. Bickerstaff (Ed.), Immobilization of Enzymes and Cells, Humana Press, Totowa, NJ, 1997, p. 193.
[32] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466.
[33] M. le Maire, J.V. Møller, P. Champeil, Biochemistry 26 (1987) 4803.
[34] D. Levy, A. Gulik, M. Seigneuret, J.-L. Rigaud, Biochemistry 29 (1990) 9480.
[35] M. Ueno, C. Tanford, J.A. Reynolds, Biochemistry 23 (1984) 3070.
[36] D. Lichtenberg, Biochim. Biophys. Acta 821 (1985) 470.
[37] K. Edwards, M. Almgren, J. Colloid Interface Sci. 147 (1991) 1.
[38] M. le Maire, P. Champeil, J.V. Møller, Biochim. Biophys. Acta 1508 (2000) 86.
[39] K. Andrieux, S. Lesieur, M. Ollivon, C. Grabielle-Madelmont, J. Chromatogr. B 706 (1998) 141.
[40] C. Engvall, P. Lundahl, J. Chromatogr. A., in press.