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Immobilized liposome and biomembrane partitioning chromatography of drugs for prediction of drug transport

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Abstract

Drug partitioning into lipid bilayers was studied by chromatography on liposomes and biomembranes immobilized in gel beads by freeze–thawing. The drug retention volume was expressed as a capacity factor, K_s, normalized with respect to the amount of immobilized phospholipid. Log K_s values for positively charged drugs on brain phosphatidylserine (PS)/egg phosphatidylcholine (PC) liposomes decreased as the ionic strength was increased, increased as the PS:PC ratio or the pH was increased and varied linearly with the temperature. Log K_s values for beta-blockers, phenothiazines and benzodiazepines on egg phospholipid (EPL) liposomes correlated well with corresponding values on red cell membrane lipid liposomes ($r^2 = 0.96$), and on human red cell membrane vesicles containing transmembrane proteins ($r^2 = 0.96$). A fair correlation was observed between the values on EPL liposomes and those on native membranes of adsorbed red cells ($r^2 = 0.86$). Compared to the data obtained with liposomes, the retentions of hydrophilic drugs became larger and the range of log K_s values more narrow on the vesicles and the membranes, which expose hydrophilic protein surfaces and oligosaccharides. Lower correlations were observed between drug retention on EPL liposomes and egg PC liposomes; and between retention on liposomes (or vesicles) and immobilized artificial membrane (IAM) monolayers of PC analogues. Absorption of orally administered drugs in humans (literature data) was nearly complete for drugs of log K_s values in the interval 1.2–2.5 on vesicles. Both vesicles and liposomes can thus be used for chromatographic analysis of drug–membrane interaction and prediction of drug absorption.

Keywords: Biomembrane chromatography; Drug absorption; Drug partitioning; Drug retention; Immobilized liposomes and biomembranes; Phospholipid bilayers

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Table 1
Biomaterials

Abbreviation	Material	Composition
PC	Phosphatidylcholine	—
PS	Phosphatidylserine	—
EPL	Egg phospholipid	Mainly PC and phosphatidylethanolamine (PE) (see Section 2.2)
Membrane lipids	Lipids extracted from human red cell membrane vesicles	Mainly PC, PE, sphingomyelin, cholesterol, glycolipids, PS (van Deenen and de Gier, 1974)
Vesicles	Cytoskeleton-depleted human red cell membrane vesicles	Mainly human red cell membrane lipids, the anion transport protein, glycophorins, and the glucose transport protein Glut1
Ghosts	Human red cell membranes	Lipids and transmembrane proteins as in the vesicles, spectrin, other cytoskeleton proteins

1. Introduction

A drug's ability to interact with cell membranes is of great importance for the behavior of the drug in the organism. For example, the pharmacokinetics, membrane permeability and access to the receptor site are often strongly dependent on interactions with cell membranes. The properties of both the drug and the membrane affect drug partitioning into the lipid bilayers (Korten et al., 1980; Welti et al., 1984; Wright et al., 1990; Ma et al., 1991; Sarmento et al., 1993; Go et al., 1995) and thus the rate of diffusion across the membrane. Liposomes of simple composition have been used as biomembrane models (Betageri and Rogers, 1988; Lundahl and Beigi, 1997) for the purpose of predicting drug transport, but natural membrane vesicles of complex composition are likely to be better models. This has been studied in the present work.

Drug interaction with immobilized artificial membrane (IAM) monolayers of phospholipid analogues covalently coupled to a silica matrix (Yang et al., 1996) and with immobilized liposomes (Lundahl and Beigi, 1997; Yang et al., 1997a,b) has been studied by chromatographic techniques. Zhang et al. (1995) used capillary electrophoresis of drugs with liposomes as a pseudostationary phase. The present work used chromatography on immobilized liposomes to reveal effects of liposome charge and composition, as well as pH, ionic strength and temperature on interactions with beta-blockers and phenothiazines. Furthermore, chromatographic retention of beta-blockers, phenothiazines and benzodiazepines was determined on liposomes composed of phosphatidylcholine, egg phospholipids or human red cell membrane lipids, as well as on cytoskeleton-depleted red cell membrane vesicles and red cell ghosts. Table 1 describes the material denotations that will be used below and shows the main components of the various materials. Correlation with drug retention on commercially available IAM.PC monolayers was studied, since columns of this type are used in drug discovery programs. Finally, the retention of thirteen structurally diverse compounds on the vesicles was related to literature values of their absorption after oral administration to humans.

2. Materials and methods

2.1. Materials (cf. Table 1)

Superdex 200 prep grade and glass columns (HR 5/2 and 5/5) were bought from Pharmacia Biotech (Uppsala, Sweden), hens' egg PC (95%) from Avanti Polar Lipids (Alabaster, AL, USA) and cholic acid ($>99\%$) from Fluka (Buchs, Switzerland). An IAM.PC column (IAM.PC.DD No. 777006), 10 cm $\times$ 4.6 mm (i.d.), with immobilized monolayers of synthetic PC analogues (Regis, Morton Grove, IL, USA) was kindly provided by Dr. S. Singh (Pharmacia and Upjohn, Uppsala, Sweden). Desmethyldiazepam, diazepam, flunitrazepam, nitrazepam and oxazepam were kindly provided by Dr. P. Smith (Smith-

Kline Beecham, PA, USA). All other drugs (see legends to the Figures) and bovine brain PS (type III extract, 80–85%) were purchased from Sigma (St. Louis, MO). All chemicals were of analytical grade.

2.2. Preparation of vesicles and lipids (cf. Table 1)

The vesicles were prepared as described by Lundahl et al. (1986). In that work the vesicles were denoted integral membrane proteins. EPL (70% PC, 21% PE, 9% other phospholipids and lysophospholipids, and small amounts of cholesterol and other components) were prepared from hens' egg (Yang and Lundahl, 1994). The membrane lipids were extracted from the vesicles by the technique described by Folch et al. (1957). Briefly, chloroform–methanol (2:1, v/v, 200 ml) and vesicle suspension (15 ml, 300 mg phospholipid) were mixed and vigorously shaken in a separation funnel. Precipitated protein was removed by filtration and the solvents were rotary evaporated. The lipid film was redissolved and reformed twice with chloroform and twice with diethylether.

2.3. Immobilized liposome and biomembrane partitioning chromatography (cf. Table 1)

Liposomes (1.5 ml, 100 mM phospholipids), prepared by rehydration of lipid films in 150 mM NaCl, 1 mM Na_2EDTA and 10 mM Tris–HCl, pH 7.4 (Brekkan et al., 1997), or the vesicle suspension, were mixed with dry Superdex 200 gel beads (110 mg) and were immobilized by gel bead swelling followed by freezing and thawing to effect liposome or vesicle fusion (Yang and Lundahl, 1994; Brekkan et al., 1996, 1997; Lundqvist et al., 1997). The material was packed into a 5-mm (i.d.) HR glass column to a 1.0-ml gel bed containing about 45 mM liposomal phospholipid or 9 mM vesicle phospholipid. The eluent was 150 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4. An HPLC pump provided a constant flow rate. Aliquots of 10–50 μl of drugs dissolved in the eluent or in ethanol and diluted with the eluent to 0.02–0.5 mg/ml ($<5\%$ ethanol) were analyzed at 0.5–1.0 ml/min at 23°C, with detection at 220 nm (Beigi et al., 1995). In special experiments, deviations from the above conditions were done, as stated below.

The percentage of phospholipids retained in the gel bed after 40–80 runs over periods of 2–8 weeks at 23°C was 50–97% of the liposome or vesicle phospholipids present before packing. However, half or more of the losses occurred during packing, and the stability during series of runs was adequate for the analyses presented here. Liposomes with high PS content showed the largest losses. Better stability can be achieved by use of biotin–avidin immobilization (Yang et al., 1997a,b). For phospholipid determination see Section 2.5.

2.4. Immobilized biomembrane partitioning chromatography of drugs on ghosts (cf. Table 1)

Human red cells from fresh A^+ blood with EDTA were immobilized in derivatized gel particles prepared from piperazine diacrylamide, methacrylamide and *N*-allyldimethylamine essentially as described earlier (Zeng et al., 1997). Washed cells (4.0×10^9) were adsorbed to suspended gel particles at room temperature for 60 min with occasional gentle agitation. The material was packed into a 1.0-cm (i.d.) plexiglass column and was washed overnight with isotonic sodium phosphate buffer containing 5 mM D-glucose and 1 mM ATP (0.1 ml/min, 4°C). The eluent was not deaerated. For drug retention analysis, the ATP was excluded from the eluent to avoid interference with drug detection at 220 nm. Under these conditions most cells probably lysed, and the stationary material was denoted ghosts for brevity. Drug aliquots of 10–50 μl (0.1–0.5 mg/ml) were analyzed at 0.1 ml/min at 23°C over a period of three weeks.

2.5. The capacity factor

For normalization of the results obtained on gel beds of different sizes and with different amounts of phospholipids, a capacity factor, K_s, for a drug was calculated by use of the equation

$$K_s = \frac{V_R - V_o}{A} \quad (1)$$

where V_R is the retention volume of the drug; V_o is the retention volume of a small and hydrophilic reference molecule ($K_2Cr_2O_7$ or NaN_3); and A is the amount of immobilized phospholipids. The units were chosen to obtain K_s in M^{-1}. The retention volumes were read at the peak maxima. Eq. (1) does not take into account the total liposome or vesicle volume or the drug interaction with the gel matrix, thereby deviating from a previously used equation (Beigi et al., 1995; Brekkan et al., 1995). However, the difference between the K_s values calculated by the two equations was negligible, except for the gel particle bed with ghosts, which required correction for drug–matrix interaction.

The phospholipid amount A was determined as the average of the phosphorus amounts (Bartlett, 1959; Sandberg et al., 1987) in the gel suspension before column packing and in the resuspended gel or the cholate-eluted lipid solution after the experiments (following thorough exchange of the phosphate in the buffer solution for Tris) or, in the case of ghosts, as the product of the number of cells in the immobilization mixture and the average phospholipid amount 3.0×10^{-13} g/cell (van Deenen and de Gier, 1974), with an approximate average molecular weight of 1000 for the phospholipids.

3. Results

3.1. Flow rate, bed volume, lipid concentration and drug concentration

The $\log K_s$ value obtained for a drug was essentially independent of the chromatographic flow rate in the range 0.1–1.2 ml/min, as shown by runs of nadolol, pindolol, alprenolol and promethazine on immobilized PC liposomes, in agreement with earlier results by Brekkan et al. (1995), indicating equilibrium between free and interacting drug molecules. Neither the bed volume (0.1–1 ml) nor the concentration of immobilized phospholipids (15–55 mM) affected the $\log K_s$ values, which indicates that Eq. (1) is applicable. The average $\log K_s$ values ($\pm$S.E.M., n = 13–16) in the entire series of analyses were: nadolol, 0.87 ± 0.10; pindolol, 1.59 ± 0.13; alprenolol, 2.42 ± 0.10; and promethazine, 3.49 ± 0.08.

Frontal chromatographic runs with application of large-volume samples (50 ml) showed no effect of drug concentration in the range of 0.002–40 μg/ml in analyses of pindolol ($\log K_s$ 1.41 ± 0.03) and alprenolol ($\log K_s$ 2.26 ± 0.08), which furthermore indicates that the zonal retention volumes were unaffected by drug concentration in the range used, since the dilution upon zone spreading during the runs was on the order of 100-fold.

3.2. Lipid charge and composition, ionic strength, pH and temperature

An increasing proportion of negatively charged PS in PC liposomes increased the retention of positively charged beta-blockers and phenothiazines on the immobilized bilayers (not illustrated), due to increasing electrostatic interaction and presumably also to other effects of the changes in phospholipid composition. The $\log K_s$ values for nadolol, pindolol, alprenolol and promethazine were, on the average, higher by the term 0.45 ± 0.08 on PS/PC liposomes with 43 mol% PS than on PC liposomes without PS (as judged by rectilinear fit to $\log K_s$ values for each drug on liposomes with 0, 18.5, 28 and 43 mol% PS). For prochlorperazine, $\log K_s$ value 3.76 on PC liposomes, the corresponding increase was 0.94.

Drug retention on PS/PC liposomes with 18.5 mol% PS, 74 mol% PC and 8% unknown components was studied as a function of ionic strength, pH and temperature (Fig. 1). The $\log K_s$ values decreased gradually with increasing NaCl concentration (Fig. 1A), consistent with electrostatic effects. The $\log K_s$ values were higher on these liposomes (solid lines in Fig. 1A) than on PC liposomes of negligible net charge (broken lines) over the entire range of NaCl concentrations. An electrostatic effect was observed also with the PC liposomes, particularly for the most hydrophilic drug, nadolol. This may be related to the electric dipole moment that is dependent on the orientation of the phospholipid headgroup (Makino et

al., 1991). The increase of $\log K_s$ values in the pH interval 9–11 (Fig. 1B) can be attributed to deprotonation of the drugs or the lipid head-groups: for example, the amine groups of PS ($pK_a \approx 10$). Similar pH effects were reported recently (Krämer and Wunderli-Allenspach, 1996). The $\log K_s$ values varied rectilinearly when the temperature was increased from 10 to 60°C. The lines for beta-blockers tended to converge, whereas the values decreased for phenothiazines, displaying individual thermodynamic features (Fig. 1C).

3.3. Drug-bilayer partitioning on biomembranes

The $\log K_s$ values for the retention of 17 drugs on EPL liposomes showed a moderate rectilinear correlation with those on PC liposomes (Fig. 2A, $r^2 = 0.79$). The correlation of the $\log K_s$ values on EPL liposomes with those on membrane lipid liposomes (Fig. 2B) and on the vesicles (Fig. 2C) was very good ($r^2 \approx 0.96$ in both cases). This was probably due to similarities in lipid compositions, PC and PE being major components, and to a substantial heterogeneity in all of the latter cases. The values on EPL liposomes showed a moderate correlation with values on ghosts ($r^2 = 0.86$, Fig. 2D) for 14 of the drugs (excluding c and g, which were eluted earlier than $K_2Cr_2O_7$ on ghosts, and o, which was eluted earlier than without ghosts).

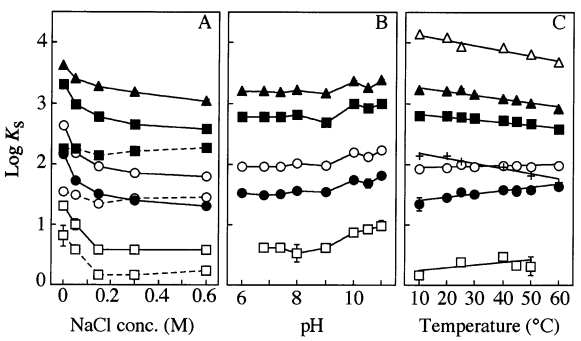

Fig. 1. Effects of ionic strength, pH and temperature on the $\log K_s$ values of drugs on PS/PC liposomes (solid lines) with a molar ratio of 18.5% PS. The broken lines in panel (A) represent $\log K_s$ values on PC liposomes. $\log K_s$ values are shown versus the NaCl concentration (A) and pH (B) in the eluent, and the temperature (C), for the beta-blockers atenolol (open square), metoprolol (filled circle), oxprenolol (open circle), alprenolol (filled square), propranolol (filled triangle), and, only in (C), the phenothiazines promethazine (open triangle) and fluphenazine (+). The eluent was 0–0.6 M NaCl in 10 mM sodium phosphate buffer, pH 7.4 (A), 150 mM NaCl in 10 mM sodium phosphate buffer, pH 6–8, or in 10 mM Na_2EDTA, pH 9–11 (B), or 150 mM NaCl in 10 mM sodium phosphate, pH 7.4 (C).

The slopes in Fig. 2A–D increased in the order $A \approx B < C < D$ (1.01, 1.15, 1.41 and 2.01, respectively), with a wide spreading of the $\log K_s$ values on the liposomes and intermediate spreading on the vesicles, whereas the narrow range of $\log K_s$ values on ghosts limited the discrimination among the drugs. The slopes probably reflect the extent of polar interactions of the relatively hydrophilic drugs with hydrophilic amino acid residues on the membrane surfaces and with the carboxylate groups of the sialic acid residues within the oligosaccharide chains of glycophorins and other proteins. The proportion of hydrophilic membrane components is highest in the ghosts with their cytoskeletons, intermediate in the vesicles with hydrophilic faces of glycosylated transmembrane proteins, and lowest in the liposomes, which contain only the limited hydrophilic regions of the bilayers.

Finally, Fig. 3 shows the correlation between the series of $\log K_s$ values on the vesicles and ghosts ($r^2 = 0.92$). The fact that the correlation was not perfect shows that removal of the cytoskeleton at high pH or other modifications of the membrane during the preparation of vesicles from ghosts affect the drug-membrane interaction.

3.4. Comparison between chromatography on IAM.PC monolayers and on liposomes or vesicles

Despite the head-group identity between the PC analogues forming monolayers in the IAM.PC column and the PC lipid bilayer liposomes, only a moderate rectilinear correlation ($r^2 = 0.83$) was observed between $\log K_s$ values for beta-blockers, phenothiazines and benzodiazepines on the PC liposomes and $\log k'_{IAM}$ values for the same drugs on the IAM.PC monolayers (Fig. 4), which supports the assump-

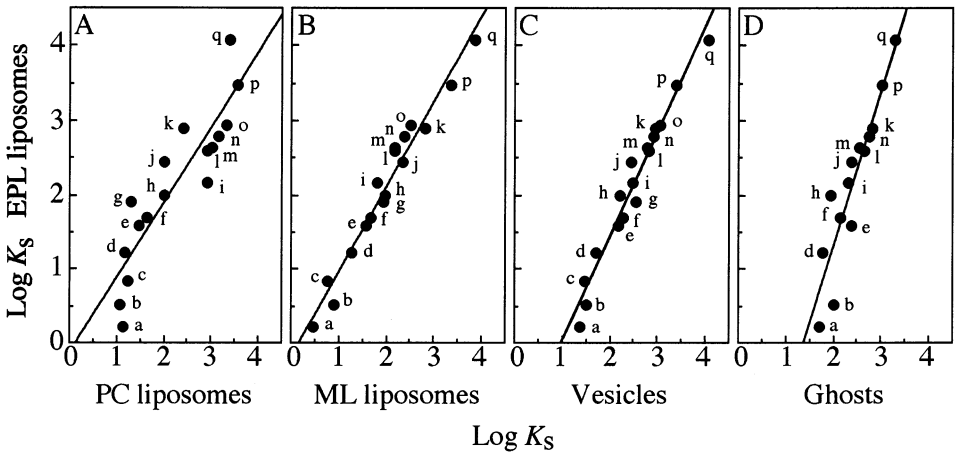

Fig. 2. Log K_s values of beta-blockers, phenothiazines and benzodiazepines on EPL liposomes versus the log K_s values on (A) PC liposomes, rectilinear correlation $r^2 = 0.79$, (B) membrane lipid (ML) liposomes, $r^2 = 0.96$, (C) the vesicles, $r^2 = 0.96$, and (D) ghosts, $r^2 = 0.86$. The drugs were a, atenolol; b, acebutolol; c, nadolol; d, metoprolol; e, pindolol; f, oxprenolol; g, fluphenazine; h, pyrilamine; i, flunitrazepam; j, alprenolol; k, propranolol; l, diazepam; m, nitrazepam; n, oxazepam; o, desmethyldiazepam; p, promethazine; and q, chlorpromazine. Average values are given for three runs on each of two gel beds (EPL liposomes, average standard error of the log K_s values ± 0.02; PC liposomes, ± 0.03, and the vesicles, ± 0.21), or on a single gel bed (membrane lipid liposomes and ghosts). The deviation was negligible between values from runs on each separate gel bed.

tion that the structures of the hydrophobic regions of the lipid layers are important. The correlations between the log k'_{IAM} values in Fig. 4 and the corresponding log K_s values obtained on EPL liposomes, membrane lipid liposomes and the vesicles were similar to that above ($r^2 = 0.83$, 0.79 and 0.81, respectively), indicating that the monolayer and bilayer systems differ substantially, independently of the bilayer composition.

3.5. Correlation between drug retention on vesicles and oral drug absorption in humans

Absorption values for drugs orally administered in humans became high at intermediate log K_s values on the vesicles (Fig. 5). A 4th degree polynomial gave an approximate fit to the data ($r^2 = 0.68$) and indicated an absorption $> 90\%$ in the interval $1.2 < \log K_s < 2.5$. A similar graph was

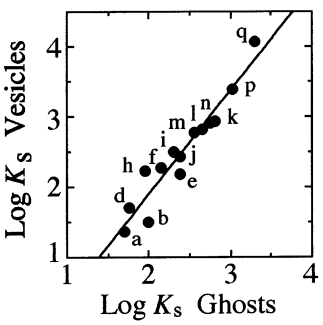

Fig. 3. Log K_s values for drugs on the vesicles versus log K_s values on ghosts. The rectilinear correlation is $r^2 = 0.92$. The drugs are denoted as in Fig. 2.

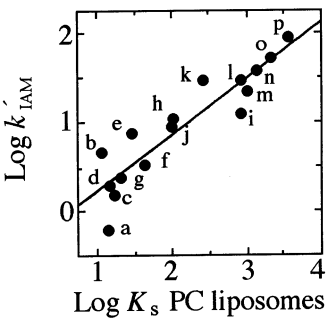

Fig. 4. The logarithms of the drug capacity factors k'_{IAM} on the IAM.PC column versus log K_s values obtained on PC liposomes. For the definition of k'_{IAM}, see Ong et al. (1996). The rectilinear correlation is $r^2 = 0.83$. The drugs are denoted as in Fig. 2.

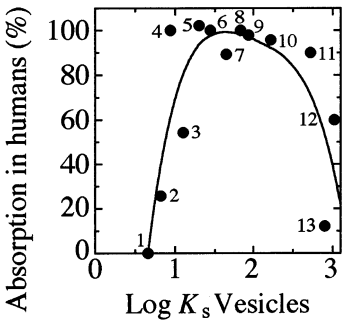

Fig. 5. Oral drug absorption in humans as listed by Palm et al. (1997) versus log K_s values obtained on the vesicles. The drugs are 1, polyethylene glycol (average M_W 200); 2, mannitol; 3, atenolol; 4, acetylsalicylic acid; 5, metoprolol; 6, salicylic acid; 7, hydrocortisone; 8, corticosterone; 9, warfarin; 10, alprenolol; 11, propranolol; 12, terbutaline; 13, sulphasalazin. A single gel bed was used to determine the log K_s values. The drugs 3, 5, 10 and 11 correspond to a, d, j and k in Fig. 2, respectively. The absorption value for terbutaline is from Fagerholm et al. (1996). The equation for the curve is $y = -390.38 + 967.75x - 711.67x^2 + 232.93x^3 - 29.106x^4$.

reported by Beigi et al. (1995) for PC liposomes. In addition to the change from liposomes to vesicles, some absorption data have been adjusted according to recent reports and data have been added (Palm et al., 1997).

4. Discussion

Drug partitioning into liposomes and biomembranes has been suggested to show better correlation with drug diffusional permeability across lipid bilayers and with drug efficacy than does partitioning into an *n*-octanol phase, owing to the structural differences between the solvent and the bilayers (Rogers and Choi, 1993; Seydel et al., 1994). The chromatographic biomembrane partitioning system used in the present study allowed the application of both model biomembranes and certain natural biomembranes for analysis of the effects of chromatographic parameters and lipid compositions. However, all of the different liposomal and biomembrane compositions, except PC, gave similar results (Fig. 2). Presumably the essential feature of a good biomembrane model for drug partitioning analyses is a bilayer heterogeneity mimicking that of natural membranes.

The presence of membrane proteins compressed the observed range of log K_s values, as the retardation of hydrophilic drugs increased (Fig. 2), presumably owing to polar drug–protein interactions. Single-point electrostatic interactions alone can hardly affect drug–membrane interactions under physiological conditions, since even strong multiple-point interactions between charged proteins and oppositely charged lipid bilayers require low ionic strength to cause adsorption (Lundahl and Yang, 1991), but cooperative effects of electrostatic interaction together with hydrophobic drug–membrane partitioning may occur. This is probably the explanation for the differences between PC bilayers and mixed PS/PC bilayers (Fig. 1A, broken and solid lines, respectively). In a similar way headgroup effects may contribute to the deviations from the lines in Fig. 2.

The correlation between the absorption of drugs in humans after oral administration (literature data) versus log K_s values on the vesicles (Fig. 5) showed low absorption at the outskirts of the range $0.6 < \log K_s < 3.0$ found for the analyzed substances, whereas drug absorption was nearly complete at intermediate log K_s values. At low log K_s values the drug is too hydrophilic for partitioning into the membrane, whereas at higher log K_s values the absorption is probably hampered by the high partitioning into the membranes and the slow diffusion out from the membrane.

The present study shows that useful retention data for prediction of drug absorption can be obtained by chromatography of drugs on immobilized liposomes or biomembranes of heterogeneous compositions. Previous chromatographic and electrophoretic studies were mainly limited to the use of egg PC and 1,2-dipalmitoyl-phosphatidylcholine liposomes (Beigi et al., 1995; Brekkan et al., 1995; Zhang et al., 1995, 1996; Yang et al., 1997b), although a few data for EPL and PS/PC liposomes and for the vesicles have been reported (Lundahl and Beigi, 1997). Studies of the relationships between chromatographic retention of drugs on model membranes and oral absorption and transport across cell membranes will lead to better understanding of the chemical properties that are important for membrane partitioning. Furthermore, these studies will also show

the potential of liposome or biomembrane partitioning chromatography for screening of drugs for absorption properties at an early stage in the drug discovery process.

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