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Immobilized-liposome chromatographic analysis of drug partitioning into lipid bilayers☆

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Abstract

The chromatographic retardation of drugs on a gel bed with immobilized liposomes was shown to correlate with the absorption of the drugs through epithelial cell layers, which is related to drug partitioning into the lipid bilayers of cell membranes. The capacity factors were divided by the lipid concentration (mM) in the gel bed to obtain specific capacity factors, K_s. The logarithm of the octanol–water distribution ratios showed a linear correlation with log K_s, whereas the logarithm of the apparent permeability coefficients in epithelial cell monolayers and the absorption of drugs orally administered in humans (data from other laboratories) increased over the interval $0 < \log K_s < 1$ and attained a saturation level in the interval $1 < \log K_s < 3$. Immobilized-liposome chromatography may be applicable for prediction of drug uptake through epithelial cell membranes.

1. Introduction

The epithelium of the human small intestine absorbs drugs through the cell membranes or the tight junctions between the cells. The rate of absorption can be studied in a human intestinal epithelial cell line monolayer model [1,2]. Drug absorption across the cell membrane is related to the hydrophobicity of the drugs in terms of their experimental octanol–water distribution ratio, D, which is defined as the ratio between the sums of the concentrations of all drug species (non-charged and charged, monomeric and oligomeric) in the organic phase and in the aqueous phase, whereas the octanol–water partition coefficient, P_{oct}, applies strictly only to non-charged drug monomers, as is discussed extensively in the literature [3]. Among numerous investigations in this field, thermodynamic studies of partitioning of chemical compounds and pharmaceutical drugs between an aqueous phase and phosphatidylcholine (PC) liposomes, cyclohexane or *n*-octanol can be mentioned [4–6]. An optical method is available for determining drug partitioning into lipid bilayers and utilizes measurements of refractive index changes in a lipid bilayer deposited onto a planar optical waveguide [7]. Partitioning of drugs into a hydrophobic phase can also be analyzed by HPLC with suitable stationary phases [8]. How-

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ever, analysis of partitioning into lipid monolayers or bilayers seems advantageous, since organic phases cannot account for the existence of specific polar interactions between the compounds and phospholipid bilayers [9]. Recently, HPLC beds with PC coatings have been used with aqueous eluents containing an organic modifier [8,10,11], particularly for drugs of high P_{oct} values. Columns containing monolayers of phospholipids covalently bonded to silica gel beads for "immobilized artificial membrane (IAM)" chromatography seemed useful in model studies related to the transport of drugs through human skin [12]. For the prediction of rates of drug transport across membranes, chromatographic analysis of the interaction of drugs with liposomes should be preferable, since liposomal lipid bilayers are structurally similar to biological membrane bilayers and suitable for experiments under physiological conditions, avoiding organic solvents as eluent modifiers for eluting the most hydrophobic drugs. Furthermore, the restraint posed by the covalent immobilization of each individual lipid molecule in the IAM monolayer does not exist in the liposomal system.

Chromatography on immobilized liposomes and proteoliposomes, for example, of proteins on liposomes ("liposome chromatography") and of glucose on glucose transporter (Glut1) proteoliposomes ("transport retention chromatography") have been described earlier [13–15]. In the present work, drugs were applied to immobilized-liposome chromatographic (ILC) columns to study the partitioning of drugs into liposomes. For this new approach, liposomes were immobilized by freeze–thawing [16] in small agarose–dextran gel beads, which were packed into columns. Pharmaceutical drugs of various structures and with a wide range of log P_{oct} values were applied to the columns and showed widely different retention volumes. The ILC results were compared with partition coefficients determined with free liposomes [19], and with data on drug absorption through monolayers of cultured epithelial cells of the cell line Caco-2 [1,2] and on absorption of orally administered doses [2].

2. Experimental

2.1. Materials

Egg PC (95% or 99%) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sephadex G-50 M and Superdex 200 HR (13-μm crosslinked agarose beads with grafted dextran) were obtained from Pharmacia Biotech AB (Uppsala, Sweden). Acetylsalicylic acid (aspirin) (A-5376), alprenolol (A-8676), atenolol (A-7655), chlorpromazine (C-8138), corticosterone (C-2505), hydrocortisone (H-4001), metoprolol (M-5391), polyethylene glycol (PEG) (P-3015), salicylic acid (S-3007), terbutaline (T-2528) and warfarin (A-2250) were purchased from Sigma (St. Louis, MO, USA). DL-propranolol (P-0884) was a gift from P. Artursson, Dept. Pharmaceutics, Uppsala University. Other chemicals were of analytical grade.

Eluent A was 10 m*M* Tris-HCl, pH 7.4, and 150 m*M* NaCl. Eluent B was 10 m*M* sodium phosphate, pH 7.4, and 150 m*M* NaCl. The eluents were passed through a 0.2-μm filter (11107-47-N, Sartorius, Göttingen, Germany) and were simultaneously degassed.

2.2. Preparation and immobilization of liposomes

Liposomes were prepared by removal of cholate from cholate–lipid solutions on Sephadex G-50 M gel beds in eluent A at 22°C, concentrated in a membrane concentrator (Minicon B15, Amicon, Beverly, MA, USA) at 4°C, mixed with partially dried gel beads and immobilized by freeze–thawing, all essentially as described in Ref. [16], except that Superdex 200 HR beads were utilized. Briefly, beads were partially dried with a water aspirator, or were ethanol-dried for the immobilization of 99% PC liposomes, and were thoroughly mixed with a liposome suspension of 180–280 m*M* phospholipid concentration. The mixture (0.5–2 ml) was vortexed and kept at room temperature ($\approx$22°C) for at least one hour to allow the liposomes to enter the swelling gel beads and

was frozen in ethanol–CO_2 (s) at −75°C and thawed at 25°C. This procedure effected steric immobilization by increasing the liposome size to dimensions similar to the spaces available in the network of the gel matrix. The beads with immobilized liposomes were left at room temperature for one hour. Nonimmobilized liposomes were removed by suspending the gel-liposome mixture in buffer A and washing five times at 20°C by centrifugation at 350 *g* for 15 min (see also Discussion). The amount of phospholipids per packed gel volume was determined by phosphorus analysis [16,17].

2.3. Immobilized-liposome chromatography (ILC) of drugs

ILC of drugs was performed on PC liposomes in Superdex 200 HR gel beads packed in 5-mm I.D. glass columns (HR 5/5 and HR 5/10, Pharmacia Biotech). Three immobilized-liposome gel beds were used: 1.0-ml bed volume, 95% PC, 53 or 63 m*M* phospholipid; and 2.0-ml bed volume, 99% PC, 43 m*M* phospholipid. The drugs were dissolved in eluent B at 0.02–10 mg/ml or were dissolved in ethanol or 0.1 *M* NaOH and diluted with eluent B to a concentration in that range. Aliquots (30–50 μl) were applied to the column and were eluted at 22°C with eluent B at a flow-rate of 0.1–0.5 ml/min with an HPLC pump (No. 2248, Pharmacia Biotech AB) with absorbance detection at 210 or 220 nm (Waters 486 detector, Millipore, Milford, MA, USA). After the ILC experiments, the liposomes were eluted with 100 m*M* cholate solution, the amount of phospholipids was determined [17], and the drugs were run on the liposome-free gel beds in order to estimate the contribution of the gel matrix to the retardation on the liposome-Superdex gel bed.

2.4. The specific capacity factor (K_s)

The retention volume, V_R, on a liposome column and the corresponding retention volume, V_0, after removal of the liposomes, were used to calculate the specific capacity factor, K_s, defined as

$$K_s = [V_R - (V_0 - V_L)]/[(V_0 - V_L)B].$$

The liposome volume, V_L, estimated by chromatography of [^{14}C]glucose before and after removal of the liposomes [18], was introduced to correct for the difference in total column volume between the columns with and without liposomes. The concentration of immobilized phospholipids, B, expressed as μmol phosphorus/ml gel bed, was introduced for combining data from the different columns. K_s was thus expressed in the unit mM^{-1}.

Strongly hydrophobic drugs showed high retardation on the liposome columns and minor retardation even on the liposome-free columns. The latter retardation was taken into account by the term V_0 in the above definition of the specific capacity factor K_s.

3. Results

3.1. Immobilized-liposome chromatography with PC liposomes

All drugs were run two to six times on each of the three immobilized-liposome columns and showed an essentially constant retention volume and a constant peak area for a given drug on a given column, which indicates that no appreciable loss of liposomes or adsorption of the drug occurred during a run. The nearly symmetric peaks showed that equilibration was essentially attained even at high flow-rate [20]. The average loss of lipids after ≈100 runs over a period of three weeks was 7%.

A typical pair of drug elution profiles in runs with and without liposomes is shown in Fig. 1. The peaks were relatively broad, especially for the drugs that were strongly retarded on immobilized liposomes. These drugs were slightly retarded even on the liposome-free columns. Other drugs showed no significant retardation on the liposome-free columns.

The log K_s values were essentially indepen-

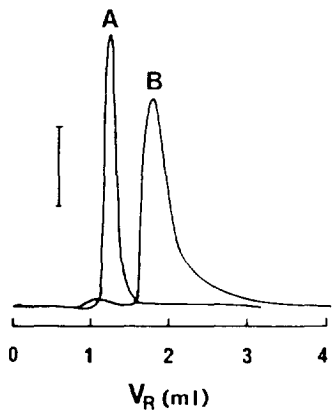

Fig. 1. Elution profiles for terbutaline on an immobilized-liposome Superdex 200 HR column (peak B) and on the same column after removal of the liposomes (peak A). Gel bed: 5 × 0.5 cm I.D. (0.98 ml). Liposome composition: PC, 99%. Phospholipid amount: 63 μmol. Eluent: B. Flow-rate: 0.1 ml/min. Terbutaline amount: 0.5 μg. The bar indicates an absorbance at 220 nm of 0.02 (peak A) and 0.01 (peak B).

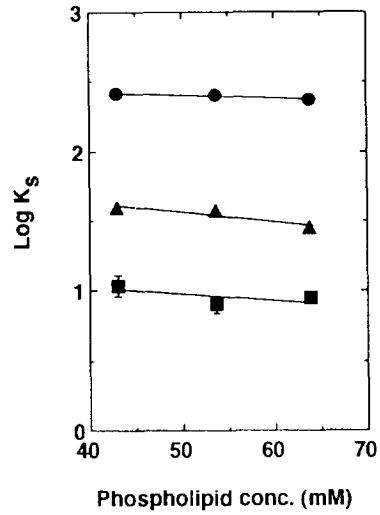

Fig. 2. The logarithm of the specific capacity factor, K_s, versus the phospholipid concentration in three ILC columns with PC liposomes (see Experimental) for two drugs of different hydrophobicities: terbutaline (■) (error bars shown) and alprenolol (●) (error bars contained within the solid circles). The average of the log K_s values on each column for terbutaline, aspirin, corticosterone, warfarin and alprenolol (▲) was also calculated (no error bars, since the drugs have widely different K_s values).

dent of the column length and the phospholipid concentrations in the gel beds in the range used, 40–65 m*M* (Fig. 2). The slightly negative slopes are barely significant, but may be due to multilamellarity of the liposomes (see Discussion).

The logarithm of the octanol–water partition coefficients (log P_{oct}) [6] showed poor correlation with the logarithm of the specific capacity factor, log K_s, (linear correlation coefficient $r \approx 0.7$; not illustrated). The retardations did not only depend only on the hydrophobicity of the drugs as reflected by their partition coefficients (log P_{oct}), since most of the tested drugs have ionizable groups with different pK_a values [6] and are charged at pH 7.4. The logarithm of the octanol–water distribution ratio *D* (the concentration ratio for all species of the drug [3]; see Introduction) correlated fairly well with the logarithm of the specific capacity factor, log K_s (Fig. 3). The linear correlation coefficient was $r = 0.94$ for the combined results from three series of determinations. The drugs tested were nonhomologous. Within homologous series of drugs a better correlation can be expected.

The series of experiments on the three columns showed almost the same pattern. An additional experiment (not illustrated) was done on immobilized PC-liposomes with a mixture of eight drugs, which separated on a 0.3-ml gel bed (0.5 × 1.5 cm) into one moderately retarded zone and three broad and strongly retarded zones, the latter ones corresponding to the three most hydrophobic drugs. On a 1.8-ml gel bed (0.5 × 9.3 cm) the first zone split into two zones. For drugs of low log *D* values a long column may be suitable for precise determination of K_s values, whereas for more hydrophobic drugs a short column saves time and reduces diffusional effects.

3.2. Correlation with permeability of epithelial cells and absorption in humans

For several drugs, the apparent drug permeability P_{app} into monolayers of cultured epithelial cells of the cell line Caco-2 has been determined by Artursson [1] and Artursson and Karlsson [2]. Their determinations showed that the absorption of orally distributed doses in-

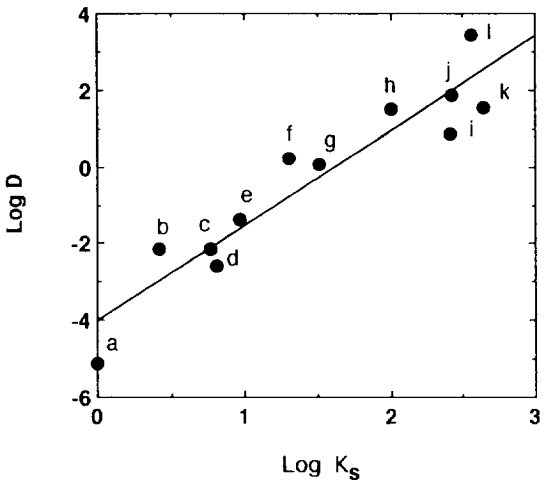

Fig. 3. The logarithm of the octanol–water distribution ratio D, versus the average log K_s value from runs of each drug on three ILC columns with different lipid amounts. The drugs were: a = PEG; b = atenolol; c = salicylic acid; d = aspirin; e = terbutaline; f = warfarin; g = metoprolol; h = hydrocortisone; i = alprenolol; j = corticosterone; k = chlorpromazine; l = DL-propranolol. The amount applied was in the range 0.5–20 μg. The log D values are from Ref. [2].

creased monotonically with increasing P_{app} values and reached 100% above $P_{app} = 10^{-6}$ cm s^{-1} [2] (Fig. 4). The absorption data also increased

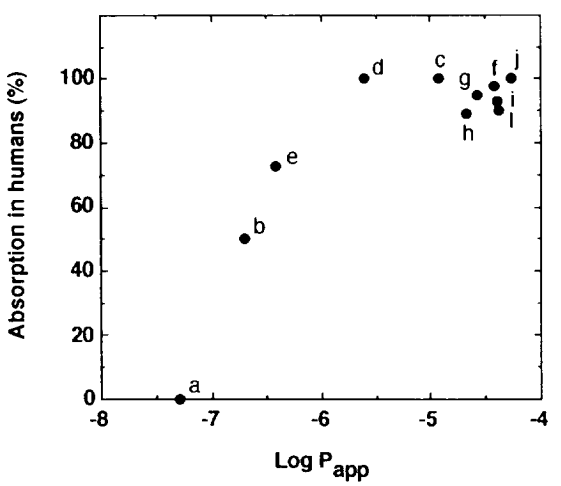

Fig. 4. Absorption of orally administered doses of drugs in humans versus log P_{app} values. Chlorpromazine (k) is excluded, other drugs are denoted as in Fig. 3. Both log P_{app} values and absorption data are from Ref. [2].

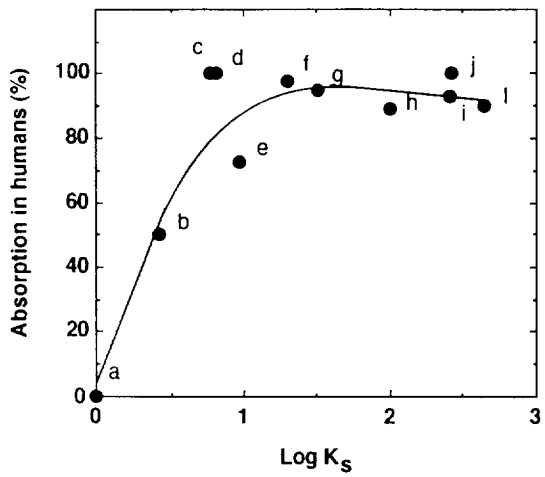

Fig. 5. Absorption of orally administered doses of drugs in humans versus the same log K_s values as in Fig. 3. The drugs are denoted as in Figs. 3 and 4. Absorption values are from Ref. [2].

with increasing log K_s values for ILC (Fig. 5), although less regularly. The absorption values tended to decrease slightly when log K_s increased from 1 to 2.7. Drugs with log $K_s > 1$ (K_s in mM^{-1}) seem to have favorable absorption properties. ILC may thus be used for the estimation of drug partitioning and drug absorption through cell membranes.

3.3. Correlation with apparent partition coefficients in fluid liposomal membranes

Apparent ion-corrected partition coefficients for β-blockers have been determined with free liposomes [19]. The corresponding non-corrected partition coefficients were calculated and compared with our K_s values from the ILC columns for the four β-blockers atenolol, metoprolol, alprenolol and propranolol (Fig. 6). The linear correlation coefficient was relatively high (r = 0.90).

4. Discussion

Superdex 200 HR gel beads were chosen for ILC, since the small and rigid beads were found to be suitable for liposome immobilization [16]

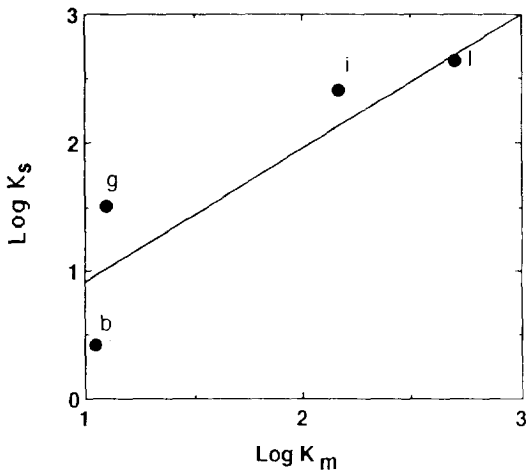

Fig. 6. Log K_s values, obtained by runs on ILC columns, versus the apparent partition coefficients determined with free liposomes [19] (without ion-correction, see text) for: b = atenolol; g = metoprolol; i = alprenolol; and l = propranolol.

and allowed a flow-rate of 0.1–1 ml/min. The PC liposomes showed good stability during the runs, although the most hydrophobic drugs tended to cause release of a minimal fraction of the immobilized liposomes, as was detected by low-angle laser light-scattering flow-cell photometry. For long-term use of a liposome column, the immobilized-liposome stability and the reproducibility of retention volumes should be checked by use of suitable standard compounds, similarly as in Fig. 5B of Ref. [20]. The columns showed good stability during runs over three weeks. By modifying the washing procedure after immobilization, a still higher stability of liposome [20] or proteoliposome (Yang and Lundahl, manuscript in preparation) immobilization was achieved. For example, a column with immobilized PC proteoliposomes was run under 45 days with a moderate loss of lipids (10%). Improved ILC columns can therefore presumably be run for at least 2–3 months with a large number of drug applications, provided that calibration with a standard drug is performed regularly. Over a long period of time, lipid degradation may affect the column performance.

The log K_s values for the drugs seemed to decrease slightly with increasing lipid concentration in the gel beds. Increasing amounts of liposomes in the bed during the freeze–thawing immobilization presumably results in increasing multilamellarity of the liposomes formed and immobilized [16], which would decrease the liposome outer surface area leading to a decrease in drug retention volume and trailing of the elution peaks caused by non-equilibrium partitioning of the drug into the internal bilayers. The small effect observed in the present experiments (Fig. 2) indicates that any differences in multilamellarity that occurred over the lipid concentration range used were of little consequence at the flow-rates employed.

Most of the drugs tested in the present study were charged at pH 7.4. The chromatographic retention of drugs expressed as the logarithm of the specific capacity factor (log K_s) correlated better with log D than with log P_{oct}. This indicates that the retardation of drugs on liposome columns reflects both hydrophobic and other properties of the drugs. The hydrophobic effect certainly plays an important role in the interaction between drugs and liposomes. Electron-spin resonance spectroscopy has revealed that drugs can be located either in the hydrophobic interior of the lipid bilayers or on the surface of the bilayers [4]. Hydrogen-bonding and other electrostatic interactions may affect drug distribution between the aqueous phase and the lipid bilayer.

The correlation between absorption of orally distributed drugs in humans and the log K_s values from the ILC experiments shows that log K_s can be related to the uptake of a drug into a lipid bilayer. The difference in the correlation between the absorption of orally administered drugs and P_{app} or log K_s presumably reflects the role of tight junctions in drug uptake through the epithelial cell layers. ILC may therefore become useful for the characterization of drugs, for example, in the screening of drug prototypes available only in small amounts, for rapid preliminary prediction of drug uptake in humans. The preparation of the columns is very simple. For high-volume screening, commercial columns will hopefully soon be available. Furthermore,

ILC seems applicable for detailed model studies of non-specific or specific interactions between drugs and biomembranes.

Future ILC studies should, for instance, reveal effects of the lipid composition and the net electric drug charge on drug partitioning into lipid bilayers. Different homologous series of drugs should be compared. Computer modelling of drug conformations upon interaction with lipid bilayers may be useful for interpretation of experimental K_s values, for example, for peptides.

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