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Immobilized liposome chromatography of drugs on capillary continuous beds for model analysis of drug–membrane interactions

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Abstract

Liposomes were immobilized in capillary continuous beds with covalently linked C_4 or C_8 alkyl ligands for chromatographic analysis of drug interaction with phospholipid bilayers, as reflected by drug retention volumes and calculated differences in interaction free energies. This procedure is a high-resolution micro-scale version of immobilized liposome chromatography for prediction of diffusion of drugs across biological membranes. The logarithm of the specific capacity factors of several structurally unrelated drugs showed a linear correlation with the logarithm of known apparent drug permeabilities through Caco-2 epithelial cell monolayers. The latter values are used for prediction of absorption of orally administered drug doses.

Keywords: Immobilized liposome chromatography; Capillary columns; Liposomes; Phospholipids; Drugs

1. Introduction

Drug partitioning into biomembrane lipid bilayers is essential for diffusion of drugs across the membranes, which is a major pathway for drug absorption. In partitioning studies, bulk oil and octadecyl silica phases have been used as membrane models, but liposomes with their polar interfaces have been considered to be a better model [1–5]. To facilitate the analysis, chromatographic systems have been designed. Compared with the phospholipid monolayers in immobilized artificial membrane chromatography [2,6,7], liposomal phospholipid bilayers in immobilized liposome chromatography (ILC) [8–10] have the advantage that they closely resemble biological membrane bilayers and constitute a two-dimensional fluid in which lipid molecules and other components are free to diffuse [11]. The "fluid" properties of the bilayer and possibly the formation of lipid microdomains [12] may affect the drug permeability.

Drug–lipid bilayer interactions have been studied on liposomes sterically immobilized in gel beads [8,9] and by liposome capillary electrophoresis with liposomes as a pseudostationary phase [13]. In the present study, continuous beds [14] were formed by polymerization in capillaries [15–17] and were used to anchor liposomes via hydrophobic ligands, similarly as previously done with beaded matrices [18–20]. The bed volume in the immobilized liposome microchromatography was as small as 10 μl, which is particularly advantageous when the phospholipids or analytes are available only in small quantities or

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are very expensive. Interactions between several drugs and the lipid bilayers were analyzed.

2. Experimental

2.1. Materials

Egg L-α-phosphatidylcholine (PC, 95%) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and fused-silica capillaries (I.D. 0.32 mm) from MicroQuartz (Munich, Germany). Sephadex G-50 M and γ-methacryloxypropyltrimethoxysilane (Bind-Silane) were obtained from Pharmacia Biotech (Uppsala, Sweden). Acebutolol, alprenolol, aspirin, atenolol, corticosterone, hydrocortisone, mannitol, metoprolol, nadolol, pindolol, propranolol, salicylic acid, terbutaline and warfarin were purchased from Sigma (St. Louis, MO, USA); oxazepam, nitrazepam, diazepam and flunitrazepam were gifts from Dr. Philip L. Smith, SmithKline Beecham (King of Prussia, PA, USA); cholic acid (>99%), methacrylamide and boron trifluoride ethyl etherate were bought from Fluka (Buchs, Switzerland); electrophoretically pure piperazine diacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulfate from Bio-Rad Laboratories (Richmond, CA, USA); 1,2-epoxybutane and 1,2-epoxyoctane from Aldrich (Steinheim, Germany). Other chemicals were of analytical grade.

2.2. Preparation of C_4 or C_8 alkyl derivatives of the continuous bed

After washing with 0.1 *M* NaOH and water the silica capillary was filled with 10% Bind-Silane in acetone and was kept at room temperature for 1 h to activate the inner wall with methacryl groups. Piperazine diacrylamide (0.145 g), methacrylamide (0.105 g) and $(NH_4)_2SO_4$ (0.065 g) were dissolved in 1.0 ml of 50 m*M* sodium phosphate buffer, pH 7.0, at 23°C. The solution was de-aerated, supplemented with 15 μl of 10% ammonium persulfate (w/v) and 5 μl of 20% (v/v) TEMED aqueous solutions, and sucked into the activated silica capillary for polymerization at room temperature overnight. The continuous bed was washed consecutively with water, acetone and ether. To derivatize the gel with alkyl chains the capillary was filled with boron trifluoride ethyl etherate (5% in diethyl ether, 15 min) and then with 1,2-epoxybutane (C_4 ligands) or 1,2-epoxyoctane (C_8 ligands) (10% in diethyl ether, 30 min). The derivatized gel was washed with ether, acetone and water. For liposome immobilization we selected gel beds on which aspirin, salicylic acid and warfarin did not separate in 10 m*M* sodium phosphate buffer, pH 7.4 (henceforth referred to as eluent A) at 10 μl/min and 23°C when applied in a mixture (4 μl, 5–10 μg/ml for each drug). Columns on which separation occurred were judged to have too high ligand density.

2.3. Liposome immobilization and chromatography

PC (0.15 g) was dissolved under nitrogen in 4 ml of eluent A supplemented with 125 m*M* cholate at 23°C. The solution was chromatographed on a Sephadex G-50 M gel bed (38×2 cm) in eluent A at a flow rate of 4 ml/min to remove cholate and obtain liposomes, similarly as described earlier [9,21]. Freshly prepared liposomes (average diameter ≈30 nm [22]) collected at the top of the liposome peak were used for immobilization, since the liposomes at the front of the liposome peak greatly increased the back-pressure of the continuous bed, whereas those at the trail gave a poor stability of immobilization, probably due to effects of remaining detergent, and since the liposomes aggregated gradually with time, thereby increasing the risk of blocking the column. A continuous bed (80–170 mm×0.32 mm) with C_4 or C_8 ligands was equilibrated with eluent A and a 50 μl aliquot of liposome suspension (35–50 m*M* phospholipid) was applied at a flow-rate of 10 μl/min which caused an increase in column back-pressure. The non-immobilized liposomes were eluted during a time period of about 20 h, whereupon the back-pressure decreased to stabilize at a level of two or three times the initial pressure in the absence of liposomes. Drugs dissolved in eluent A were applied (4 μl, 15–30 μg/ml) onto the immobilized liposome continuous bed and were eluted at 10 μl/min at 23°C with detection at 220 nm (UV monitor Model 200, Linear Instruments, Reno, NV, USA, modified for on-capillary detection at the workshop of the Biomedical Center, Uppsala University) (Fig.

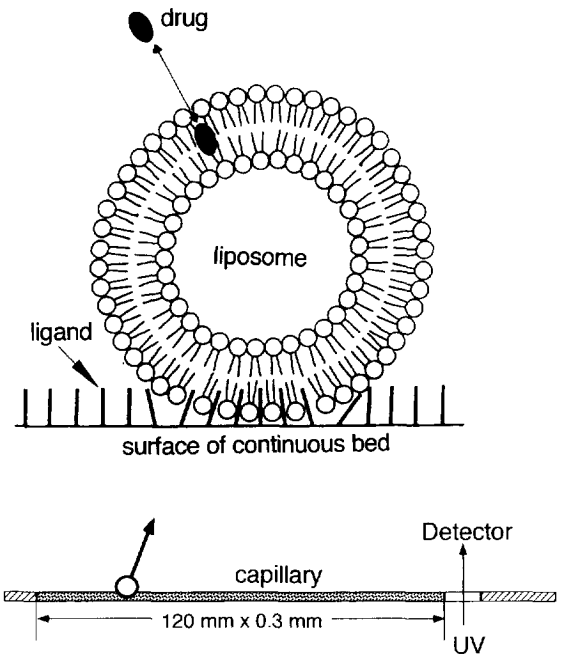

Fig. 1. Schematic illustration of the immobilized liposome stationary phase, formed by hydrophobic interaction between the liposomes and alkyl ligands coupled to the matrix of continuous beds at the surfaces of the bed channels or pores, and the set-up for analysis of drug–liposome interaction on an immobilized liposome capillary continuous bed.

1). After the chromatographic experiments, the phospholipid amount of the immobilized liposomes was determined by phosphorus assay [23] of the eluate obtained by application of 200 m*M* cholate, pH 8. To estimate the initial amount of immobilized phospholipids the gel bed was further washed with acetone and buffer, liposomes were again immobilized and the cholate-eluted phosphorus was determined.

3. Theory

By definition, the activity a_i of a solute (i) is related to the chemical potential μ_i by the equation:

$$\mu_i = \mu_i^0 + RT \ln a_i = \mu_i^0 + RT \ln f_i \cdot c_i \quad (1)$$

where f_i is the activity coefficient, c_i the concentration, R the gas constant, 8.314 J deg^{-1} mol^{-1}, and T the temperature. In chromatography, the chemical potential in the stationary phase ($\mu_{i,s}$) is equal, at equilibrium, to that in the mobile phase, ($\mu_{i,m}$), i.e.,

$$\mu_{i,s}^0 + RT \ln f_{i,s} \cdot c_s = \mu_{i,m}^0 + RT \ln f_{i,m} \cdot c_m \quad (2)$$

Assuming that the activity coefficient f_i has the same value in the two phases we obtain the following relationships:

$$\mu_{i,s}^0 - \mu_{i,m}^0 = -RT \ln \frac{c_{i,s}}{c_{i,m}} \quad (3)$$

and the difference in standard free energy, ΔG_i^0, for the solute in the stationary and the mobile phases, is

$$\Delta G_i^0 = -RT \ln K_i \quad (4)$$

where $K_i = c_{i,s}/c_{i,m}$ is the partition coefficient.

Since $K_i = (V_{e,i} - V_0)/V_i$, where $V_{e,i}$, V_0 and V_i are the retention volume, void volume and inner volume, respectively, ΔG_i^0 values can be calculated from the following equation:

$$\Delta G_i^0 = -RT \ln \frac{V_{e,i} - V_0}{V_i} \quad (5)$$

In Eq. (5) effects of pressure, interfacial tension, electric potential and adsorption [24] are neglected. The inner volume in our case includes the volume of the liposomes and is technically difficult to determine accurately, but is constant for a given immobilized liposome gel bed. If two solutes (drugs) (1 and 2) are analyzed on a single column at constant chromatographic conditions, the difference in retention volumes reflect the difference in the interaction with the lipid bilayers. The difference in standard free energy, $\Delta(\Delta G^0)$ for the interaction of solute 2 and solute 1 with the liposomes can be calculated by use of Eq. (6), where the inner volume term is cancelled and where V_0 can be determined by using a solute that is not retarded:

$$\Delta(\Delta G^0) = -RT \ln \frac{V_{e,2} - V_0}{V_{e,1} - V_0} \quad (6)$$

It may be convenient, for example, in quantitative structure–activity relationship (QSAR) studies of drugs, to evaluate partitioning by using the $\Delta(\Delta G^0)$ value of a drug relative to that of a reference drug of

known biological activity and partition coefficient [1,3].

4. Results

Aspirin, salicylic acid, warfarin and pindolol were retarded to different extents on liposomes immobilized in a C_4 bed, but were co-eluted in the absence of liposomes (Fig. 2). On the immobilized liposomes in a C_8 bed a similar elution profile was observed, but with the C_8 ligands some drugs were slightly retarded in the absence of liposomes (not illustrated). In both C_4 and C_8 continuous beds immobilization of liposomes was achieved even at low ligand density, as in gel bead beds [18], and the ligands do not significantly affect the liposome structure as judged by earlier results [18–20]. The ILC analysis in capillary continuous beds was extended to several drugs (Table 1). The retention of a drug was expressed as the specific capacity factor K_s (M^{-1}),

$$K_s = \frac{V_e - V_0}{V_0 \times B} \quad (7)$$

where V_e is the retention volume, V_0 is the void volume of the immobilized liposome bed and B is the concentration of immobilized phospholipids, expressed as mmol phosphorus/ml gel bed. Mannitol interacted with liposomes more weakly than did other analytes, with the same elution volume in several consecutive runs, and was used as a reference analyte to determine the V_0 value.

The apparent drug permeability (P_{app}) through Caco-2 epithelial cell monolayers, which are used as a model to study passive drug absorption, showed a good correlation with the absorption values in humans for orally administered doses of drugs (Fig.

Fig. 2. Elution profile of aspirin, salicylic acid, warfarin and pindolol (from left to right) on liposomes immobilized via C_4 ligands in a capillary continuous bed (length 120 mm; ≈1.0 μmol phospholipid). For details see Section 2.3. The peaks were identified by runs with different drug concentration ratios. In the absence of liposomes the drugs were eluted in a single sharp peak at the retention volume indicated by an arrow.

Table 1
Retention volumes[a], log K_s[b] and the difference in standard free energies, [$\Delta(\Delta G^0)$][c] of drugs on liposomes immobilized with C_4 ligands in a capillary continuous bed

Drug	V_e (μl)	log K_s	$\Delta(\Delta G^0)$ (kJ mol^{-1})
Mannitol	10.2±0.0	–	–
Terbutaline	10.48±0.5	−0.68	–
Atenolol[d]	10.50±1	−0.65	−0.2
Aspirin	11.2±1	−0.12	−3.1
Hydrocortisone	20.2±0.3	0.88	−8.8
Metoprolol[d]	25.2±3	1.05	−9.8
Salicylic acid	31.5±3	1.21	−10.7
Nadolol[d]	45.0±2	1.42	−11.9
Propranolol[d]	49.6±0.8	1.47	−12.2
Alprenolol[d]	50.9±2	1.49	−12.3
Oxazepam	53.9±3.8	1.52	−12.4
Corticosterone	55.3±0.5	1.53	−12.5
Acebutolol[d]	67.0±1	1.63	−13.1
Warfarin	68.6±5	1.64	−13.1
Nitrazepam	68.6±1.3	1.65	−13.2
Diazepam	75.6±13.5	1.69	−13.4
Flunitrazepam	100±2.5	1.83	−14.2
Pindolol[d]	123±6	1.93	−14.8

[a] The phospholipid amount in the capillary continuous bed (length 100 mm) was ≈1.3 μmol. V_e values measured on different columns were normalized according to the retention volumes of reference drugs. Other details are given in the Experimental section 2.3.
[b] Calculated according to Eq. (7).
[c] Calculated according to Eq. (6). Terbutaline was used as reference drug.
[d] β-Adrenoreceptor antagonist (β-blocker).

2 in Ref. [25] and Fig. 4 in Ref. [8]). The specific capacity factors (Table 1) reflect the degree of drug–liposome interaction, which, in turn, is related to the partitioning of drugs into biological membranes. A linear correlation ($r=0.97$) between the $\log K_s$ values and the available $\log P_{app}$ values (Fig. 3) suggests that the K_s values can be used similarly as P_{app} values for prediction of drug partitioning into membranes.

The $\Delta(\Delta G^0)$ values relative to terbutaline at the experimental temperature $T=296$ K were calculated by Eq. (6) and are also listed in Table 1 (see Section 5).

The amount of immobilized liposomes (1.0±0.3 μmol phospholipid in 10 μl beds) was similar to the amounts earlier reported on gel bead columns with hydrophobic ligands [18,19]. The total loss of lipids was less than 5% during one week of continuous running. The reproducibility of the retention volumes was checked by repeated runs of nitrazepam during five days of continuous running (Fig. 4). The V_e values decreased greatly between the run following immediately after the liposome immobilization and the run after one-day washing with the eluent, probably due to the loss of residual non-immobilized liposomes. Over-night washing with eluent is thus necessary. From the second day, the V_e value of nitrazepam decreased by only 5% during continuous running of several drugs.

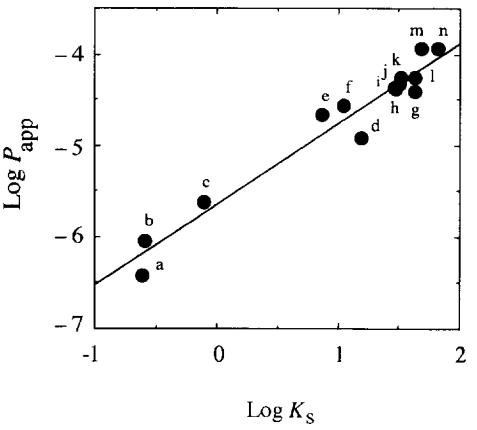

Fig. 3. Log P_{app} values versus log K_s values determined on liposomes immobilized in a capillary continuous bed (length 100 mm; 1.3 μmol phospholipid) for (a) atenolol, (b) terbutaline, (c) aspirin, (d) salicylic acid, (e) hydrocortisone, (f) metoprolol, (g) warfarin, (h) alprenonol, (i) propranolol, (j) corticosterone, (k) oxazepam, (l) nitrazepam, (m) diazepam and (n) flunitrazepam. The P_{app} values were obtained from Ref. [25] (a–j) or from Dr. Philip L. Smith, SmithKline Beecham (personal communication) (k–n).

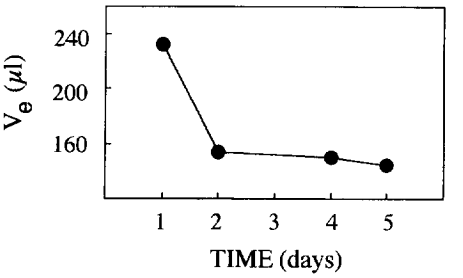

Fig. 4. The retention volumes (V_e) of nitrazepam versus the time period of continuous running at 10 μl/min on an immobilized liposome capillary continuous bed (length 170 mm; 1.0 μmol phospholipid). The average values from duplicate runs were used (average error limit ±2.5 μl).

5. Discussion

The linear correlation between $\log K_s$ and $\log P_{app}$ values for structurally unrelated drugs (Fig. 3) validated the assumption that liposomes immobilized in a continuous bed can be used as a model to evaluate or predict the partitioning or absorption properties of drugs. The specific capacity factors calculated from the retention volumes reflect the analyte–membrane interaction, in which both hydrophobic interaction and headgroup effects [26–28] are involved. On the other hand, the measured $\log K_s$ values of β-blockers on egg PC-liposomes (fatty acid composition: palmitoyl/stearoyl/oleoyl/linoleoyl; molar ratio ≈3:1:2.8:1.6) (Table 1) did not correlate with the logarithms of the apparent partition coefficients (K'_m) given in Table 1 of [1] and in Table 3 of [3] in which PC-liposomes with different combinations of dimyristoylPC, dipalmitoylPC, dicetylphosphate and cholesterol were employed. This shows that drug partitioning into liposomal membranes is affected by the phospholipid composition of the liposomes [1–3] and the compositional similarity between the model liposomes and the target membrane for given drugs should therefore be considered. The membrane bilayer is not a simple hydrophobic phase and can be mimicked meaning-

fully only when the lipid composition is suitable. ILC analysis may provide a convenient method to investigate the effects of headgroups and membrane composition. From the practical point of view it is advantageous that ILC analysis can be performed on a micro-scale by use of capillary continuous beds.

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