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Short communication

Immobilized biomembrane chromatography of highly lipophilic drugs

Farideh Beigi, Per Lundahl*

Department of Biochemistry, Biomedical Center, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden

Abstract

Drug interaction with lipid bilayers was quantified by immobilized biomembrane chromatography on a series of columns containing different small amounts of human red cell membrane vesicles to extend and characterize this technique, which shows a potential for drug screening and prediction of drug absorption in humans. The chromatographic retention volume for each drug was essentially proportional to the amount of immobilized lipid, and the slope equalled the capacity factor (K_s) previously determined on single columns. Gel beds containing 0.5–2 μmol of membrane phospholipid allowed analysis of drugs with log K_s values of 2.5–4.3 in time periods of 1 min to 1 h. Highly lipophilic drugs could thus be analyzed conveniently in aqueous buffer.

Keywords: Immobilized biomembranes; Lipophilicity; Membranes; Pharmaceutical analysis; Phospholipids; Phenothiazines; β-Blockers

1. Introduction

To predict drug diffusion across cell membranes by physicochemical screening, model partitioning systems like octanol–water [1–4] and liposome–water [1,5–8] are commonly used. Immobilized artificial membrane (IAM) lipid monolayers [9] and the hydrophobic ligands in reversed-phase columns [10,11] have served as models in chromatographic analyses of drug interactions with membrane-like materials. The IAM monolayers can be used also for adsorption of enzymes or receptors [12,13]. Proteoliposomes and red cell membrane vesicles immobilized in gel beads, e.g., by freeze–thaw entrapment, have been employed for quantitative analyses of biospecific interactions with membrane proteins [14].

To apply a biomimetic model for characterization of drugs, particularly with regard to drug absorption in humans, we have analyzed their interaction with liposomes and membrane vesicles by immobilized liposome and biomembrane chromatography (ILC and IBC, respectively) [15–18]. Liposomes immobilized by biotinylated-lipid–avidin binding have recently been used for this purpose [19]. The partitioning of drugs into the lipid bilayers of liposomes or membrane vesicles was presented in terms of capacity factors, K_s, whereby higher lipophilicity resulted in higher K_s values. These studies describe the stability of the immobilized materials during a long period of time, screening of drugs on liposomes and biomembranes of different compositions, and effects of pH, ionic strength and temperature.

The fact that drugs show widely different lipophilicities results in very long elution times for

*Corresponding author. Tel.: +46-18-4714459; fax: +46-18-552139.

E-mail address: per.lundahl@biokem.uu.se (P. Lundahl)

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highly lipophilic drugs on columns that are well suited for hydrophilic drugs. To limit the elution time the amount of vesicles or liposomes in the column should be adapted to the lipophilicity range of the analyzed drugs, or columns with different amounts of vesicles/liposomes should be used. ILC and IBC columns containing relatively large amounts of lipids make the analysis of highly lipophilic drugs time-consuming and give large zone broadening or even undetectable zones. The objective of the present work is to show that IBC columns containing small amounts of phospholipids (on the order of 1 μmol) are applicable for analysis of highly lipophilic drugs. This is important, since aqueous eluents are used to mimic a physiological situation. It is neither desirable nor possible to use organic modifiers, which will solubilize the membranes.

To prepare the stationary phase, cytoskeleton-depleted red cell membrane vesicles were immobilized by freeze–thawing of vesicles mixed with dry gel beads [20]. This natural membrane material provided good results previously [18] and can easily be immobilized to give low lipid concentration and high stability.

2. Experimental

2.1. Materials

Superdex 200 prep grade and 5 mm (I.D.) glass columns (HR 5/2) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden); Superdex 200 HR gel beads were a gift from the same company. Alprenolol, propranolol, promethazine, chlorpromazine and prochlorperazine were purchased from Sigma (St. Louis, MO, USA). Cytoskeleton-depleted human red cell membrane vesicles ('integral membrane proteins') were prepared as described in [21] and are called membrane vesicles below.

2.2. Immobilization of membrane vesicles

Membrane vesicles were immobilized essentially as described in [20,22]. In brief, Superdex 200 prep grade gel beads were ethanol-dried. Dry beads (110 mg) were mixed with membrane vesicle suspension (1.5 ml). The mixture was degassed, kept at 23°C for 3 h for swelling of the beads, and freeze–thawed (−75°C, 25°C) and vortexed five times. Nonimmobilized vesicles were removed by centrifugal washings (6×3 min at 350 *g*, 20°C) in 10 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, and 1 m*M* Na_2EDTA. The beads were suspended in the buffer solution to a volume of 5 ml.

2.3. Immobilized biomembrane chromatography

Aliquots of suspended Superdex 200 prep grade gel beads (34 μm) containing immobilized membrane vesicles were packed to beds of 0.35±0.2 ml (n=10) in the central section of a total bed of 0.54±0.13 ml (n=10) to obtain uniform drug–vesicle interaction over the entire cross-section of the vesicle bed. Accordingly, vesicle-free Superdex 200 HR gel beads (13 μm) were first packed into the HR glass columns to a 3–10 mm high gel section. Superdex 200 prep grade gel with immobilized membrane vesicles was then packed on top of the HR gel to obtain 3–40 mm of vesicle bed, and vesicle-free HR gel (3–10 mm) was finally packed on top. The column length would otherwise have affected the elution volume, since a proportion of the vesicles dependent on the geometry of the end-pieces would have escaped contact with the drug zone at the top and bottom of the vesicle beds. This approach is usually unnecessary but increased the precision of the data in Fig. 2.

The eluent was 150 m*M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.4. Samples 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 a flow-rate of 0.50 or 1.00 ml/min at 23°C with detection at 220 nm at a full-scale absorbance of 0.05–1.5 for 1-cm light path (Waters 486 detector, Millipore, MA, USA) [15]. The cell volume was 8 μl. The results below are based on altogether ≈300 chromatographic runs on ten vesicle columns and ten vesicle-free columns.

2.4. Capacity factor, K_s

The retention volume depended on the drug interaction with the membrane vesicles, the amount of vesicle phospholipid (and other lipids, particularly cholesterol, and proteins, present in proportion to the phospholipid amount) in the column, and on the drug interaction with the gel matrix. A capacity factor, K_s,

essentially independent of vesicle amount and matrix interaction, was calculated similarly as reported in [18] by use of Eq. (1)

$$V_R - V_0 = K_S A + C \quad (1)$$

where V_R is the retention volume of the drug, V_0 is the retention volume of the drug on a vesicle-free column of the same design and dimensions as for the immobilized-vesicle column, K_s is the slope, A is the amount of immobilized phospholipids, and C is the intercept (ideally zero). The phospholipid amount was determined by phosphorus microanalysis, as described by Bartlett [23], of the lipids eluted with cholate after a series of experiments, as described in [15,24]. The K_s values were expressed in M^{-1} for calculation of log K_s values.

3. Results

The broadening of the drug zones by the vesicle-

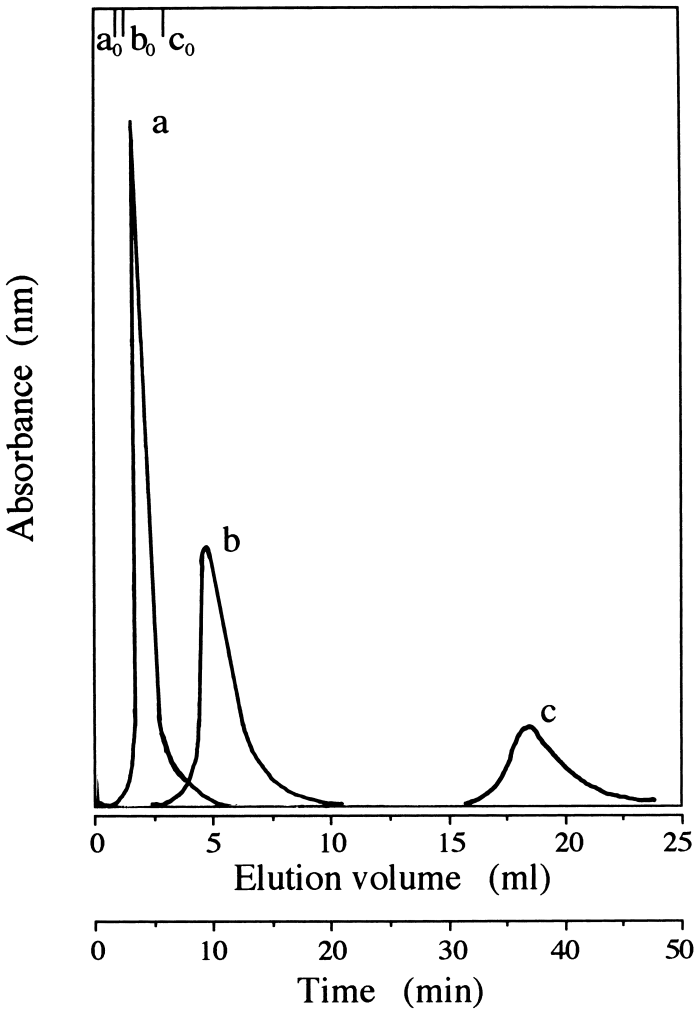

Fig. 1. Typical elution profiles of three drugs on an IBC column containing membrane vesicles. Bed volume: 0.43 ml. A central 0.29-ml section contained the vesicles (1.42 μmol phospholipids). Flow rate: 0.5 ml/min. The drugs (a) propranolol, (b) promethazine, and (c) chlorpromazine were detected at 220 nm. The lines a_0, b_0 and c_0 indicate the elution volumes of (a), (b) and (c), respectively, on a vesicle-free column of the same size and construction. The increasing dilution with increasing partitioning into the membrane is illustrated.

free sections of the small bead gel bed (see Section 2.3) was negligible as revealed by runs on vesicle-free columns (not shown). In the vesicle columns, partitioning of a lipophilic drug into the bilayers delayed the elution and caused diffusional spreading. This was apparently a major cause for peak broadening. Typical elution profiles illustrating the elution volumes and peak widths are shown in Fig. 1.

The elution volume difference, $V_R - V_0$, increased linearly with the phospholipid amount for the drugs in different membrane vesicle columns, in agreement with Eq. (1) (Fig. 2). The log K_s values obtained in the present work by use of Eq. (1) (Table 1) agreed well with the log K_s values obtained on a single immobilized membrane vesicle column containing 9 μmol of phospholipids (Fig. 2C in [18]). The small differences are probably due to the error in the determinations of the amount of membrane vesicle phospholipid. Cholesterol was presumably present in the same proportion as in the red cell membrane [25], i.e., in an amount corresponding to 80% (w/w) of the phospholipids of average molecular mass 750 [26]. The effect of the cholesterol on the drug retardation has not been quantified. For the effect of the integral membrane proteins, see Fig. 2B and C in [18].

Table 1
Comparison of (a) the present (Fig. 2) log K_s values and (b) previously reported (Fig. 2C in [18]) log K_s values

Drug	Log K_s		ΔLog K_s
	a	b	a–b
Alprenolol	2.49	2.44	0.05
Propranolol	2.91	2.94	–0.03
Promethazine	3.42	3.39	0.03
Chlorpromazine	3.95	4.06	–0.11
Prochlorperazine	≥4.3	N.d.[a]	–

[a] N.d.; not determined.

4. Discussion

The low-lipid vesicle columns containing 0.2–3.4 μmol phospholipid were useful for analyses of the

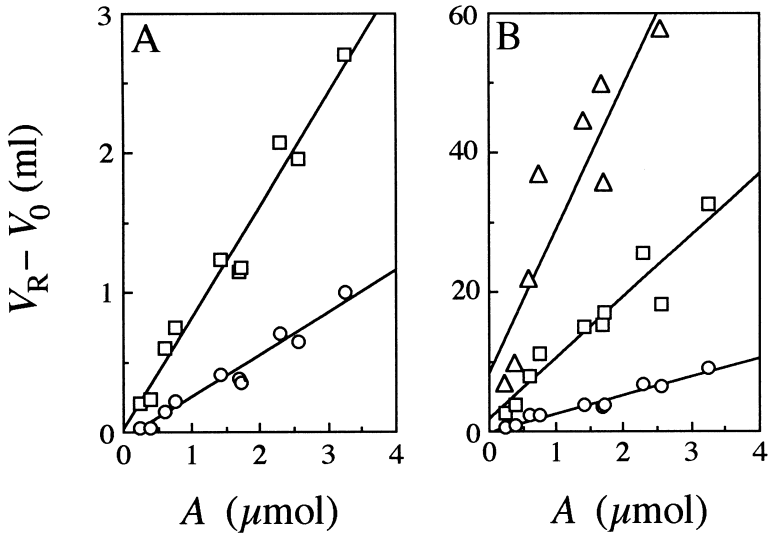

Fig. 2. Retention volume differences, $V_R - V_0$, for five lipophilic drugs run on IBC columns and corresponding vesicle-free columns versus the amount of phospholipids A in the membrane vesicles immobilized in the IBC columns. The drugs were run 2–3 times on each of the immobilized low-lipid vesicle columns. Prochlorperazine (triangles) could not be detected in runs on 3.4 μmol phospholipid column. Rectilinear curves were fitted to the points and the slopes K_s were determined according to Eq. (1). (A) Alprenolol (circles) with $V_R - V_0 = 305.38A - 0.06$ ($r^2 = 0.955$), and propranolol (squares) with $V_R - V_0 = 805A + 0.009$ ($r^2 = 0.965$). (B) Promethazine (circles) with $V_R - V_0 = 2616A + 0.08$ ($r^2 = 0.959$), chlorpromazine (squares) with $V_R - V_0 = 8843A + 1.801$ ($r^2 = 0.910$), and prochlorperazine (triangles) with $V_R - V_0 = 20843A + 8.372$ ($r^2 = 0.820$).

interaction between highly lipophilic drugs and lipid bilayers. Lipophilic drugs partition strongly into the phospholipid bilayers and will be eluted at large retention volumes in broad zones from columns containing a large amount of phospholipids [12,18]. In the present approach very lipophilic drugs could be analyzed by IBC provided that the lipid amount in the column was low. This allowed rapid analyses and drug zones could be detected that otherwise might have become too broad. For even faster analyses the chromatographic set-up can be scaled-down, in principle as for the liposomes immobilized by hydrophobic interaction in capillary continuous bed columns [27]. Packing of gel beads containing low concentrations of immobilized liposomes or vesicles in capillaries may give a robust and stable system for fast screening. Drugs of intermediate or low lipophilicity require analysis on columns with more lipids. A set of small-diameter columns or capillaries with a series of lipid amounts may be set up for automatic runs, where each substance can be run first on the bed with the lowest amount of lipid, then on a bed with more lipid, etc., ending when the elution volume exceeds a preset value that gives sufficient accuracy.

For the purpose of predicting drug uptake, data obtained by chromatographic interaction analysis should be compared with data obtained by use of other methods, such as determination of permeability across cultured epithelial cell monolayers [28] or calculation of the dynamic polar van der Waals' surface area [29,30]. The chromatographic approach seems promising for relatively fast screening of drug–membrane interaction.

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