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Immobilized liposome chromatography to study drug–membrane interactions
Correlation with drug absorption in humans

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Abstract

For rapid screening of drug–membrane interactions and predicting drug absorption in vivo, unilamellar liposomes were stably immobilized in the pores of gel beads by avidin–biotin binding. Interactions of a diverse set of well-described drugs with the immobilized liposomal membranes were reflected by their elution profiles. The membrane partitioning coefficients (K_{LM}) of the drugs were determined from the retention volumes. The drug retentions on egg phosphatidylcholine (EPC)–phosphatidylserine (PS)–cholesterol (chol) and EPC–PS–phosphatidylethanolamine (PE)–chol columns intended to mimic small intestine membranes were similar, although the positively-charged drugs were more strongly retarded on the negatively-charged liposomes than the negatively-charged drugs. The relationship between log K_{LM} with the drug fraction absorbed in humans showed that the log K_{LM} values obtained with unilamellar liposomes can be used to predict drug passive transcellular absorption, similarly to that previously shown for entrapped multilamellar liposomes. The immobilized liposome chromatography method should be useful for screening compounds at an early stage of the drug discovery process. The avidin–biotin immobilization of the liposomes prolongs the lifetime of the columns.

Keywords: Immobilized liposome chromatography; Drug–membrane interactions; Pharmaceutical analysis; Drugs; Liposomes

1. Introduction

Orally administered drugs are introduced into the human body across the epithelial cells of the digestive tract, particularly the small and large intestine. They also proceed across the brain endothelial cells of the blood vessels (BBB). Absorption of orally administered drugs in the intestine (uptake of the drug through the cell membrane) is the first step in drug action. Therefore, in the screening process for development of new drugs it is very important to estimate drug passage across the cell membrane along with the possibility of decomposition by drug-metabolizing enzymes. Drugs are absorbed through epithelial cell layers by diffusion across the lipid bilayer of the cell membranes by transport via membrane proteins (transcellular pathway), through

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the paracellular pathway, or by transcytosis. In vivo investigation of the fraction of the drug absorbed in humans (Fa) and animals are time-consuming and difficult to perform; therefore, there is a need for simple in vitro models, which may help in the differentiation between these absorption processes. Fa can be predicted using Caco-2 cells [1–3], tissue diffusion [4], liposome partitioning [5], immobilized artificial membrane (IAM) chromatography [6], immobilized liposome chromatography [7–9], parallel artificial membrane permeation assay [10], surface plasmon resonance (SPR) biosensors [11] and computational methods [12,13].

Immobilized liposome chromatography (ILC) is a recently innovated method for rapid and precise analysis of solute–membrane interactions [7,8]. In order to construct a homogeneous membrane stationary phase which mimics fluid lipid bilayer membranes, we recently immobilized unilamellar liposomes in gel beads by avidin–biotin specific binding [14], which stabilized the immobilized liposomes greatly and prolonged the column lifetime over 1 year. Such an ILC column was used for chromatographic measurement of the membrane partitioning coefficient (K_{LM}) of drugs [15,16], and analysis of the thermodynamic quantities for solute–membrane binding [17]. In addition, a fluorescent dye-entrapped liposome column was used to rapidly and sensitively detect weak solute–membrane interactions [18]. Phospholipase A_2 (PLA_2)-catalyzed membrane leakage [19] was used to detect environmental contaminants, such as polychlorinated biphenyls (PCBs) using a competitive immunoreaction [20].

In this paper, a set of chemically diverse drugs was applied onto the liposome column. In order to predict drug absorption in vivo, the K_{LM} values were compared with published data on human intestinal drug absorption, similar to Refs. [8,9]. The avidin–biotin immobilized liposome column, which has long-term stability or is simple and reproducible for estimating the membrane partitioning coefficient, can be used for predicting drug absorption in humans.

2. Experimental

2.1. Materials

Sepharose 4B (denoted Sepharose) was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. Egg yolk phosphatidylcholine (EPC, >99%), egg phosphatidylethanolamine (PE, >99%), brain phosphatidylserine (PS, >99%), and 1,2-dioleoyl-phosphatidylethanolamine-*N*-(cap biotinyl) (biotin-cPE) were obtained from Avanti Polar Lipids (Alabaster, USA). From Dojindo Labs. (Kumamoto, Japan), we obtained 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes). Egg-white avidin (M_r 66 000) was obtained from Bio-Rad (Hercules, CA, USA).

The following pharmaceutical drugs (Table 1) were supplied by Sigma (St Louis, MO, USA): acebutolol, acetaminophen, alprenolol, amoxicillin, ampicillin, antipyrine, aspirin, atenolol, cephalexin, chloramphenicol, cimetidine, clonidine, corticosterone, coumarin, diclofenac, diltazem, hydrocortisone, levofloxacin, metoprolol, oxprenolol, pindolol, progesterone, DL-propranolol, salicylic acid, sulfasalazine, terbutaline, theophylline, timolol, and warfarin.

2.2. Preparation of gel-bead-immobilized unilamellar liposomes

Avidin was coupled to CNBr-Sepharose 4B according to the procedures described in Ref. [21]. The gel contained about 3 mg avidin per ml gel bed as determined using modified Bradford assays [22], and was stored at 4 °C in buffer H (10 m*M* Hepes, 150 m*M* NaCl, 0.1 m*M* Na_2EDTA, pH 7.4) supplemented with 3 m*M* NaN_3 before use. Small unilamellar liposomes (SUVs) containing 2 mol% biotin-cPE and various kinds of lipids in the presence of buffer H were prepared by probe sonication as described in Ref. [14]. The mean size of the SUVs was 22±8 nm. For immobilization [14], the biotinylated SUVs were gently mixed with moist avidin-gel of Sepharose by rotation for 2–3 h at 23 °C or overnight at 4 °C under nitrogen. The amounts of immobilized liposomes in the gel beads were determined as phosphorus as described by Bartlett [23] with modification as described by Yang et al. [24].

2.3. Chromatography of drugs on the liposome column

The gel beads containing avidin–biotin immobil-

Table 1
Measured log K_{LM} values and drug classification data

Drug	M_r[a]	Charge at pH 7.4	Fa[b]	Tran[c]	Log K_{LM}		
					EPC	EPC–PS–chol (7:2:1)[d]	EPC–PS–PE–chol (5:1:2:2)[d]
Acebutolol	336.4	+	89.5	t	1.23	1.39	1.33
Acetaminophen	151.2	+	80	t	0.95	0.90	0.78
Alprenolol	249.3	+	93	t	2.45	2.68	2.67
Amoxicillin	365.4	±	82.5	a	−0.27	0.12	−0.33
Ampicillin	349.4	±	45	a	0.04	0.12	−0.33
Antipyrine	188.2	−	100	t	0.44	0.52	0.43
Aspirin	180.2	−	100	p	0.20	0.59	0.43
Atenolol	266.3	+	54	t	0.44	0.67	0.42
Cephalexin	347.4	±	95	a	0.21	0.12	0.01
Chloramphenicol	323.1	+	90	t	2.28	2.17	2.06
Cimetidine	252.3	+	60	a	0.89	0.90	0.74
Clonidine	213.2	+	95	t	1.14	1.31	1.31
Corticosterone	364.0	0	100	t	2.64	2.48	2.36
Coumarin	146.2	−	100	t	1.61	1.55	1.48
Diclofenac	318.1	−	99	t	3.14	2.79	2.68
Diltiazem	414.5	0	92	t	2.55	2.60	2.55
Hydrocortisone	362.0	0	91	t	2.21	2.09	1.98
Levofloxacin	361.4	+	80	t	0.44	0.59	0.51
Metoprolol	267.4	+	95	t	1.35	1.53	1.54
Oxprenolol	265.3	+	90	t	1.80	1.98	1.99
Pindolol	248.3	+	90	t	1.68	1.84	1.33
Progesterone	314.5	0	91	t	2.91	3.44	3.34
Propranolol	259.3	+	90	t	2.89	3.11	3.13
Salicylic acid	138.0	−	100	p	0.95	0.82	0.73
Sulfasalazine	398.4	−	13	t	3.16	2.77	2.64
Terbutaline	274.3	+	73	t	1.12	1.33	0.42
Theophylline	180.2	−	98	t	0.58	0.59	0.51
Timolol	316.4	+	90	t	1.54	1.66	1.65
Warfarin	308.3	−	98	t	1.62	1.36	1.20

[a] M_r, molecular mass.
[b] Fa, drug absorbed of an orally administered dose in humans [1,3,10].
[c] Tran, transport mechanism: t, passive transcellular; p, passive paracellular; a, active transport.
[d] Lipids molar ratio.

ized liposomes were packed into a 5–5.5 cm×5 mm I.D. gel bed in a glass column (HR 5/5, Pharmacia Biotech). The columns were placed in a column oven (CO-8020, Tosoh) equipped with a sample injector. Several drugs (1 μg/μl, 5 μl) were applied to the immobilized-liposome gel bed and eluted with buffer H at a flow-rate of 0.3 ml/min at 25 °C. The chromatographic runs were carried out using a Tosoh HPLC system 1 equipped with an HPLC pump (CCPM-II, Tosoh), a UV detector (UV-8010, Tosoh) set at 220 nm, and a recorder, which was interfaced with an IBM computer.

2.4. Membrane partition coefficients measured by ILC

The membrane partition coefficient for the solute partitioning between the mobile and stationary liposome membrane phase can be calculated according to [15–17]:

$$K_{LM} = (V_r - V_o)/0.755A \quad (1)$$

where V_r is the retention volume of the drug, V_o is the retention volume of a small and hydrophilic mole-

cule ($K_2Cr_2O_7$) on the liposome column (ml), and A is the amount of immobilized liposomes in the column (mmol of phospholipid). The derivation of this equation has previously been described in detail [14,17].

3. Results and discussion

A diverse set of well-described drugs (column 1, Table 1) has been selected because reliable estimates of the fraction absorbed in human were available. The drugs used in this study were classified by their transport mechanism across the intestinal cell (column 5, Table 1). Most drugs were absorbed passively by a transcellular route (t); three drugs based on aspirin, theophylline and salicylic acid, with a molecular mass below 200, were assumed to be mainly absorbed by a paracellular route (p). Amoxicillin, ampicillin and cephalexin are mainly absorbed by an active transport mechanism (a).

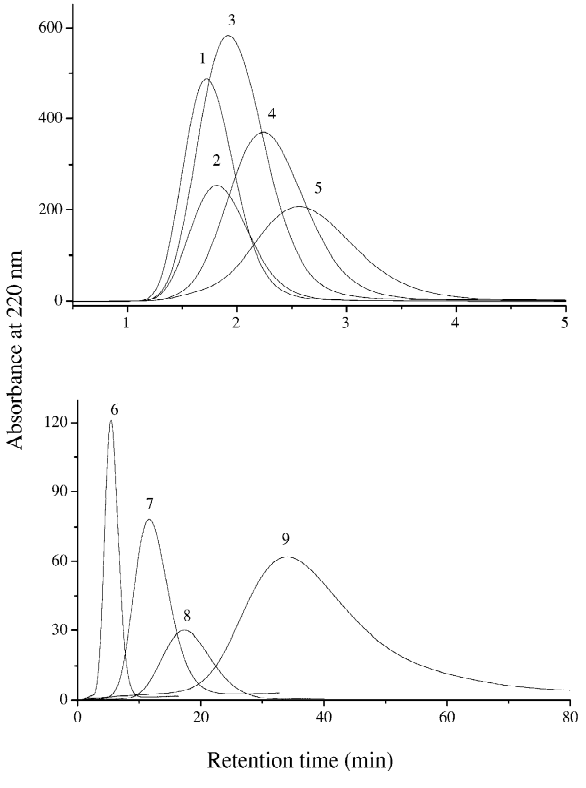

Fig. 1. Elution profiles of the drugs on an EPC–PS–chol liposome column (20 mm×5 mm I.D.). The amount of immobilized phospholipid was 10 μmol. Curves 1–9 correspond to the elution profiles of $K_2Cr_2O_7$, theophylline, cimetidine, clonidine, metoprolol, chloramphenicol, diltiazem, diclofenac, and DL-propranolol, respectively. The flow-rate was 0.3 ml/min.

Sepharose gel with a very hydrophilic network composed of polysaccharide agarose [25] was used in this study. It has been demonstrated that the non-specific binding of solutes on this gel matrix is lower than that on other matrices, such as Sephacryl or TSK gels [17]. A typical ILC chromatogram is shown in Fig. 1. Curve 1 shows a reference analyte using $K_2Cr_2O_7$. Different drugs were retarded to a different extent on the liposome column (curves 2–9, Fig. 1). As a control, drugs showed no retardation on a liposome-removed Sepharose column (data not shown). It was again demonstrated that the drug retardation on the liposome column is caused by their interaction with the immobilized liposomal membrane [16,17], but not the gel matrix. We note that such elution profiles (Fig. 1) of the drugs have not been demonstrated either with IAM chromatography [6] or with ILC [7–9]. Therefore, drug retention on the unilamellar liposome column enables chromatographic measurements of the drug–membrane partitioning coefficient as presented in Section 2.4.

As shown in Table 1, the drugs were applied to columns containing three kinds of liposomes composed of EPC, EPC–PS–chol and EPC–PS–PE–chol. Compared to the log K_{LM} values on the EPC column, the log K_{LM} values on the negatively-charged liposome columns were higher for most positively-charged drugs, but lower for negatively-charged drugs, e.g. warfarin, coumarin, diclofenac or sulphasalazine (columns 6–8, Table 1). These results agree with those in the literature [9,11,26] and show that electrostatic interaction between the ionized drugs and the negatively-charged liposome surfaces are involved in drug–membrane partitioning. A good correlation ($r=0.99$) was observed between log K_{LM} values obtained on an EPC–PS–chol column and that on an EPC–PS–PE–chol column (linear regression).

In our previous work [15], it was found that negatively-charged and cholesterol-containing liposomes are suitable models for the ILC analysis of drug–membrane interaction. In addition, Chapelle

and Gilles-Baillien [27] and Brasitus et al. [28] have reported that the lipid compositions of small-intestine brush-border membranes are EPC, PS, PE and cholesterol. Therefore, an EPC–PS–PE–chol model may be suitable to predict drug intestinal absorption. Fig. 2 shows the correlation between the log K_{LM} values obtained on an EPC–PS–PE–chol column and the fraction absorption in humans (Fa). Open circles in this figure correspond to drugs being passively absorbed via the transcellular routine (column 5, Table 1). The moderately and highly absorbed compounds are separated from each other, and a sigmoidal relationship with Fa could be seen. However, the actively transported drugs (filled triangles, Fig. 2) and some drugs having $M_r < 200$ (open triangles, Fig. 2), where the paracellular diffusion route is possible, were identified as outliers. The open square for sulphasalazine, which shows a large deviation, may be explained by efflux [13], similarly to the results for sulphasalazine obtained by others [9,11]. Antipyrine (filled circles, Fig. 2) has a high Fa value, but a low log K_{LM} value, similar to the results measured by an SPR biosensor system [11]. It should be noted that our log K_{LM} values

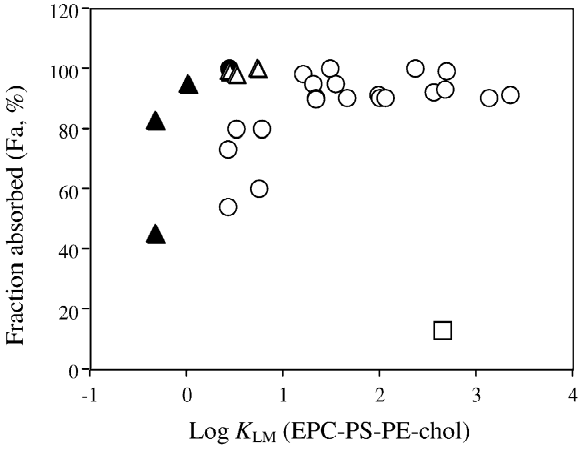

Fig. 2. Relationship between log K_{LM} on EPC–PS–PE–chol (molar ration 5:1:2:2) and drug oral fraction absorbed (Fa) in human. From left to right: ○, atenolol, terbutaline, ofloxacin, cimetidine, acetaminophen, warfarin, clonidine, pindolol, acebutolol, coumarin, metoprolol, timolol, hydrocortisone, oxprenolol, chloramphenicol, corticosterone, diltiazem, alprenolol, diclofenac, DL-propranolol, and progesterone; △, aspirin, theophylline and salicylic acid; ▲, amoxicillin, ampicillin and cephalexin; •, antipyrine; □, sulfasalazine.

(column 7, Table 1) correlated well ($r = 0.95$, $n = 10$) with the SPR biosensor results (column 6, Table 1 in Ref. [11]). Therefore, the behavior of the majority of well-absorbed drugs (Fa > 70%) would be correctly predicted with respect to in vivo passive transcellular absorption by log K_{LM}. The paracellularly absorbed drugs having a log $K_{LM} < 1.0$, or $M_r < 200$, seem to be difficult to classify by this method. A combination of our method with a Caco-2 cell monolayer measurement may provide further information on the degree of drug absorption and transport routes.

Correlation of the chromatographic retention volumes, normalized as K_s for drugs on immobilized liposomes, with drug absorption in human has been shown using sterically immobilized multilamellar liposomes [7–9]. Although there are many areas of agreement between the present and the previous work [7,26], the two advantages of the unilamellar liposome column should be mentioned here. First, the membrane partitioning coefficients (log K_{LM}) of drugs can be easily and reliably measured by ILC using large unilamellar liposomes. Unilamellar liposomes expose all of their membrane surfaces for interaction with drugs, thus having an advantage over multilamellar liposomes by avoiding the equilibration of the drug between the multiple lamellas. Second, avidin–biotin immobilized liposomes have excellent stability as previously reported [15,17,18]. There was only 1% lipid loss after chromatographic runs of 29 drugs on the same liposome column over a period of 1 month. Moreover, the log K_{LM} values for 29 drugs show almost no difference between two chromatographic runs of each drug on the same liposome column 1 year apart.

4. Concluding remarks

The liposome column can be used for preliminary drug screening and for in vitro prediction of Fa in human for drugs that follow the transcellular passive transport route. Therefore, a single chromatographic parameter, log K_{LM}, can be used to predict drug absorption at an early stage of the drug discovery process. Some pharmaceutical companies use a high throughput screening system based on Caco-2 cells. The two systems are complementary, and the present system can be used for about 1 year, while the

system using Caco-2 must be renewed for every assay. Recently, many researchers have focused on drug-absorption by transporters, but many drugs are absorbed by passive diffusion. Therefore, the present method seems useful for actual estimation of drug absorbance in the drug-developing process.

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