Determination of Liposome Partitioning of Ionizable Drugs by Titration

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Abstract □ Drug partitioning to liposomes has been suggested as a model for partitioning to biomembranes but has been lacking a rapid analytical assay useful for drug screening. A fast pH-metric titration method for the determination of liposome partitioning of ionizable drugs using small unilamellar phosphatidylcholine vesicles prepared by sonic homogenization has been successfully developed, enabling the use of high lipid-to-drug ratios. Liposome–water partition coefficients of diclofenac and propranolol were determined to study the impact of varying titration parameters, temperature, equilibration time, lipid, and liposome types on the partitioning. To validate this method, the results were compared to literature values generated with different techniques and to pH-metric titration results with large unilamellar vesicles. The rapid pH-metric assay gave liposome partitioning data for the two model compounds which were consistent with other analytical techniques and liposome types.

Introduction

Partitioning between phospholipid bilayer vesicles and water has been suggested as a better model for predicting the passive diffusion of drug molecules through biomembranes than partitioning between organic bulk solvents and water.[1−4] In contrast to octanol and other water-immiscible solvents no bulk phase separation between aqueous and organic component occurs in liposome systems. Therefore, standard spectrophotometric techniques cannot be routinely applied to measure drug partitioning in liposomes. The combination of equilibrium dialysis with radiotracer assays has been successfully used to measure partitioning at drug concentrations far below saturation of the lipid and can therefore be regarded as a gold standard for the determination of liposome partitioning.[5] However, this method fails to be useful as a routine method.

Approximately 75% of all drugs are bases, 20% are acids, and less than 5% are nonionic.[6]

The Sirius semiautomated pH-metric logP titration equipment offers a less cumbersome means of generating liposome partitioning data on a routine basis.[7] The impact of titration variables on pH-metric partitioning results with liposomes has been largely unknown and is studied here (e.g., temperature, titration direction, equilibrium time, pH-range). The liposomes are tested for titratable impurities as could, for example, be generated by hydrolysis of phospholipids.

The choice of lipid is studied together with the impact of different liposome preparation techniques and resulting vesicle sizes.

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The major components of biomembranes such as intestinal brush border membranes are phospholipids, neutral lipids, and proteins. The bilayer has been suggested to be the primary barrier to passive transcellular drug absorption. A contribution of membrane-based proteins to this mode of absorption has not been reported. The molar ratio of rabbit brush border membrane lipids is reported to be 1:1:1 (neutral:phospholipid:glycolipid).[8] The distinct bilayer configuration of the zwitterionic phosphatidylcholine in vesicles appears to mimic the interfacial character as well as the ionic, H-bond, dipole−dipole, and hydrophobic interactions which may define partitioning in real biomembranes. The impact of cholesterol, the most important neutral membrane lipid, is discussed later on.

Small unilamellar vesicles are prepared by sonication, allowing for particularly high lipid concentrations (e.g., 100 mg phospholipid/mL) and consequently for high molar phospholipid/drug ratios (above 100), avoiding saturation of phospholipid with drug. The minimal vesicle size should provide for a maximum surface area, allowing for minimal partitioning equilibrium times. Also, this type of liposome can be prepared with simple lab equipment in sufficient quality and in short time. pH-metric partitioning data from sonicated small unilamellar vesicles (S-SUV) are compared to reference values from small unilamellar vesicles as prepared by equilibrium dialysis (ED-SUV) and other published data. Furthermore, they are compared with other partitioning data obtained by the Sirius pH-metric technique with large unilamellar vesicles prepared by a freeze and thaw technique (FAT-LUV).

Experimental Section

Materials—Diclofenac sodium and propranolol hydrochloride were obtained from Sigma. Cholesterol was obtained from Fluka (Sigma Chemie Vertriebs GmbH Deisenhofen, Germany). Epikuron200 (Soy-PC: >92% glycerophosphocholine esters with various mainly unsaturated fatty acids, <3% lyso-phosphatidylcholine, <2% other phospholipids, <0.8% water, <2% oil, and <0.2% α-tocopherol) was provided by Lucas Meyer GmbH (Hamburg, Germany). Linoleic acid was the predominant fatty acid of the 70−80% of unsaturated fatty acids. Lipoid PC 18:1; 18:1 containing 98.5% dioleoylphosphatidylcholine (DOPC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Potassium chloride, HCl−Titrisol, and KOH−Titrisol were purchased from Merck KGaA (Darmstadt, Germany).

Liposome Preparation—S-SUV Liposomes were prepared as follows: 1.6 g of phospholipid was dissolved in a small amount of methanol in a 200 mL round-bottom flask. A solid phospholipid film was formed by vacuum evaporation of methanol at 50 °C using a rotary evaporator. As determined by thermogravimetry, the resulting film contained less than 5% volatiles after 30 min of vacuum drying. The phospholipid film was dispersed with 14.4 mL of a 0.15 M potassium chloride solution resulting in a phospholipid concentration of 100 mg/mL. A 15 mL aliquot of this phospholipid dispersion was sonicated for 20 min in a Sirius-titrator vial using a Bandelin Sonopuls HD70 sonifier equipped

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Table 1—Lipid Ratios Used for Investigations on the Impact of Assay Variations on S-SUV Partitioning Results

[lipid]:[drug][a]	lipid (g)	0.15 M KCl (mL)	"lipid/water"[b]
3	0.025	20	0.0013
10	0.1	15	0.0067
40	0.2	8	0.0250
100	1	20	0.0500

[a] [lipid]:[drug]: molar ratio of lipid to drug. [b] "lipid/water": lipid to water volume ratio as calculated based on partial densities for the aqueous and phospholipid moiety at 1.0 g/mL.

Table 2—Lipid Ratios Used for Investigations on the Effect of Vesicle and Lipid Types on Partitioning

[lipid]:[drug][a]	lipid (g)	0.15 M KCl (mL)	"lipid/water"[b]
1.5	0.005	15	0.0003
3.0	0.025	20	0.0013
10	0.1	15	0.0067
30	0.2	8	0.0250

[a] [lipid]:[drug]: molar ratio of lipid to drug. "lipid/water": lipid to water volume ratio as calculated based on partial densities for the aqueous and phospholipid moiety at 1.0 g/mL.

with a TT13 sonotrode (Bandelin Electronic GmbH, Berlin, Germany) at 50% amplitude setting with argon purge and cooling with ice−water.

Vesicle Size—The size of the SUV liposomes was measured by photon correlation spectroscopy with a Coulter N4 Plus at 90° angle and 600 s sample time. The evaluation of the results could be limited to a range from 5 to 500 nm. Samples were diluted with 0.2 μm filtered 0.15 M KCl solution to reach 10^5 to 10^6 counts per second. Liposomes were not filtered to avoid artifacts.

Determination of Partition Coefficients—Titrations were performed on PCA101 and GLPKA automatic titrators (Sirius Analytical Instruments Ltd., Forest Row, UK). All titrations were carried out in the pH range between 3.5 and 10.5. Alkalimetric titrations from pH 3.5 to 10.5 were called "up-assay", and acidimetric titrations from pH 10.5 to 3.5 were called "down-assays" and differed only with respect to titration direction. In this pH range drug concentrations >0.4 mM were required. After sonication, the liposome dispersion was added manually to the weighed diclofenac sodium or propranolol hydrochloride samples in 20 mL vials. Different volume ratios of lipid dispersion and water were adjusted according to the scheme in Table 1 by automatic addition of 0.15 M KCl solution. A partial density of 1.0 g/mL for aqueous KCl solution and for the phospholipid[9] in the liquid-crystalline state was used for the calculation of the lipid to water volume ratio. For the comparison of S-SUV and FAT-LUV vesicles, the mixtures were constituted according to Table 2. Samples were stirred until complete dissolution of the drug. The resulting diclofenac−liposome solutions had a pH of 7, and the propranolol−liposome solutions a pH between 5.6 and 6.6. Then the pH was adjusted automatically to the start pH of the titration at pH 10.5 or 3.5. The maximum titrant volume increment for one titration step was limited to 0.01 mL. The pH change per titrant addition was limited to 0.2 pH units. Typically, more than 30 pH readings were collected from each titration. The pH values were recorded when the pH-drift was lower than 0.01 pH per minute. The titration time was between 20 and 60 min for low and high lipid concentrations, respectively. Argon purge was applied to all titrations. Processing of titration data was carried out using the PKALOGP version 5.01 software (Sirius Analytical Instruments Ltd., Forest Row, UK). Final partitioning results log $P_{neutral}$ and log P_{ion} were calculated from titrations at different lipid concentrations including at least the two extremes of the lipid-to-drug ratio. A detailed description of the pH-metric log P method can be found elsewhere.[7,10,11]

Results and Discussion

The impact of assay conditions on drug partitioning into small unilamellar soybean phospholipid bilayer vesicles was studied in detail and is discussed in the following paragraphs. Table 3 shows the impact of different assay parameters on the partitioning of neutral and anionic diclofenac and neutral and cationic propranolol. Table 4 shows the results of a study on titratable lipid impurities measurable at the assay conditions. Table 5 shows the comparison of results as generated with the preferred titrimetric assay conditions (see line 2) with results of different liposome and lipid types and analytical techniques. Table 6 shows the results of a reproducibility study.The chemical structures and pK_a values of the neutral species of the two model drugs are shown in Figure 1.

Direction of Titration—The neutral species of diclofenac and propranolol showed no significantly different partitioning for down-titrations. The partitioning of the propranolol cation was found to be reduced by 0.5 log units for the down-titration while no different partitioning was found for the diclofenac anion (see Table 3, columns 1 and 2). The data quality of the results obtained from the down assays was generally better and resulted in smaller errors for the calculated partition coefficients. For this reason the down-assay was selected for routine measurements.

Lipid−Drug Ratio—Columns 2−4 of Table 3 show the differences of partitioning results when calculated based on four titrations covering lipid−drug ratios from 3 to100 or based on three titrations at lipid−drug ratios 3−40 or 10−100. We found no significant differences in the final results when ratios as small as 3 were used together with high ratios.

Temperature—The application of the pH-metric technique to liposome partitioning did not show any evidence for analytical problems or critical liposome instabilities at 37 °C so that this physiologic temperature can be used for routine application. Also, the higher temperature should accelerate the adjustment of the partitioning equilibrium. No significant partitioning differences were found for the different ionic species of the two model drugs at standard laboratory temperature 25 °C and physiologic temperature 37 °C (see Table 3, columns 2 and 5). The pK_a of propranolol was shifted to a significantly lower basicity at the higher temperature, which is typical for many bases, and points out the necessity to evaluate the partitioning and pK_a at the same temperature (see Figure 1).

Equilibration Time—The results of titrations with 3 h extra equilibration time before starting the titration are shown in Table 3. The partitioning of the propranolol species remained unchanged. The partition coefficients of diclofenac acid and anion rose by 0.4 and 0.5 log units, respectively. Another assay variation included a 2-min delay time after each titration step. The additional equilibration time during the assay led to nonsignificant changes of partitioning results. The minimal impact of the additional delay time suggests such a prolongation of the assay is not necessary for a rapid and continuous titration.

Titration pH-Range—The usable pH-range for titrations with phospholipids is limited by ionizable functions in the liposomes and degradation of phospholipids at extreme pH-values. It was found that the ionization of the lipid phosphate disturbs the assay at a pH lower than 3.5. The upper limit of the titration pH-range is defined by increasing hydrolytic degradation of phosphatidylcholine to titratable free fatty acids. Typically, the pH of the sample dissolved in liposome dispersion is approximately neutral. The automatic titration program quickly approaches the acidic or alkaline start pH and approaches the neutral region again after 10 to 30 min depending on the lipid and sample concentration. Consequently, the liposome stressing conditions at extreme pH-values prevail only shortly. With start pH-values not higher than 10.5, assays could be evaluated without major disturbance by generated degradation products or other artifacts.

Table 3—Impact of Assay Variations on S–SUV Partitioning Results[a]

temperature (°C)	25	25	25	25	37	25	25	25
direction of titration	up	down	down	down	down	down	down	down
lipid	Soy-PC	Soy-PC	Soy-PC	Soy-PC	Soy-PC	Soy-PC	Soy-PC	Soy-PC/cholesterol 1:1 (molar)
equilibrium time before assay	0	0	0	0	0	0	3 h	0
equilibrium time after titrant addition	0	0	0	0	0	2 min delay	0	0
range [lipid]:[drug]	3-100	3-100	3-40	10-100	3-100	3-100	3-100	3-100
				log P_{S-SUV}				
diclofenac neutral	4.7	4.5	4.3	-	4.3	4.6	4.9	3.8
diclofenac ion	2.9	3.0	2.9	-	2.9	3.0	3.5	2.7
Δ (neutral–ion)	1.8	1.5	1.4	-	1.4	1.6	1.4	1.1
propranolol neutral	3.5	3.4	3.3	3.3	3.2	3.3	3.4	3.0
propranolol ion	3.1	2.6	2.6	2.5	2.5	2.4	2.8	2.2
Δ (neutral–ion)	0.4	0.8	0.7	0.8	0.7	0.9	0.6	0.8

[a] The pH-titration was performed either from pH 3.5 to 10.5 "up" or in the opposite direction "down". "Range [lipid]:[drug]" gives the range of lipid ratios of Table 1 which were used for the multiset calculation of final log *P* results as listed in the lower section of the table.

Table 4—Titratable Impurities[a]

lipid (g)	total volume (mL)	approx pK_a of impurity	concn (mM)	mol % (impurity/total lipids)
0.025	20	6.4	0.042	2
0.1	15	6.8	0.074	1
0.2	8	6.7	0.192	0.6
1.0	20	6.8	0.185	0.3

[a] The molar fraction of titratable impurity was calculated as oleic acid (mw 282).

Titratable Impurities—An impurity, probably a fatty acid with an apparent pK_a at 6.8,[12] could be detected in blank titrations containing only the phospholipid vesicles without any drug compound. Blank titrations were carried out at 37 °C as down-assays (see Table 4). The calculated concentrations of impurity for the assays with high phospholipid concentrations were more reliable than those at lower phospholipid concentrations since concentrations of impurities below 0.1 mM were likely to be too low to be determined exactly. Partitioning of drugs into phospholipid bilayers has been reported to be influenced by concentrations of free fatty acids above 10%.[13] The direct influence of acidic impurities on the titration curves can be corrected by the refinement of data. Although the start of the titrations at pH 10.5 may increase the formation of these hydrolysis products, the measured free fatty concentration was still below the concentration reported for biological membranes.[8] High free fatty acid values should be interpreted carefully as membrane preparation techniques could also lead to phospholipid decomposition. Generally nonsaturated phospholipids such as soybean phospholipids are susceptible to autoxidation and might form peroxides. For minimizing oxidation, the sonication was carried out under argon at low temperature, and the titration was performed under argon. The titration of an aged liposome dispersion (8 h at 25 °C) did not show additional titratable impurities so that oxidation of the phospholipid is regarded to be insignificant.

Lipid Selection—The liposomes in this study were prepared from a purified soybean phosphatidylcholine so that titratable biomembrane constituents such as free fatty acids, phosphatidylethanolamine, and phosphatidylserine were minimized. The pattern of predominantly unsaturated fatty acids in soy-phospholipids provides for a fluid bilayer structure at 25 °C or higher temperatures. Compared to pure, chemically defined phospholipids such as dioleoylphosphatidylcholine (DOPC), which consists of >98.5% DOPC, phospholipids of natural origin have the advantages of a fatty acyl chain composition similar to biomembranes and low price. No significant differences were found for the partitioning of neutral propranolol and diclofenac to Soy-PC and DOPC liposomes. In contrast, the difference (Soy-PC-DOPC) was up to 0.8 for the diclofenac anion and 0.5 for the propranolol cation. However, the partitioning results for propranolol in Soy-PC/S−SUV were found to be closer to the reference results based on Egg-PC/ED-SUV than those based on DOPC/S−SUV or DOPC/FAT-LUV.

Influence of Cholesterol—Small unilamellar vesicles with cholesterol were made from a 1:1 molar mixture of Soy-PC and cholesterol.[14] The presence of cholesterol in liposomes decreased partitioning for all species, but the decrease for the diclofenac anion may not be significant (see below). The difference between log $P_{neutral}$ and log P_{ion} (Δ (neutral−ion)) was not significantly changed compared to cholesterol-free liposomes (see Table 3). A similarly significant decrease of partitioning with raising cholesterol concentrations in a bilayer was reported for nimodipine by Mason et al.[15] The titrimetric measurement of partitioning to cholesterol containing vesicles appears to be viable and may lead to further refined assay procedures.

Effect of Vesicle-Size—The size determination of the Soy-PC-S-SUV liposomes yielded a mean diameter of 32 ± 2 nm resulting in a vesicle surface area of approximately 35 m^2 per 100 mg of lipid. In contrast, the FAT-LUV technique leads to larger liposomes with a diameter of approximately 100 nm.[7] For the neutral species, no significant changes in partitioning were observed for different vesicles sizes (see Table 5). The diclofenac anion partitioning was decreased in small vesicles by 0.4 log units in DOPC and Soy-PC. The propranolol cation partitioning was decreased by. 0.5 log units in DOPC. The decrease of ion partitioning with lower vesicle size was found to be slightly smaller for Soy-PC based vesicles compared to DOPC. Small vesicles with their stronger curvature have been regarded as a less celllike membrane model than larger liposomes. On the other hand, the measurement in highly concentrated small vesicle dispersions allows for measuring at higher lipid concentrations, higher lipid/drug ratios, and maximum interfacial surface area. This benefit may outweigh the impact of higher artificial curvature.

Reproducibility—The reproducibility of titrimetric liposome partitioning results was assessed at 25 °C based on down-titration results at four different lipid concentrations according to Table 1 which were generated by three operators in two different labs. The results of each four-titration set were fitted to obtain one weighted average partition coefficient for the different ionic species of the two test compounds as listed in Table 6. The high reproducibility of the partitioning results could be achieved despite some differences in equipment, e.g., new versus old sonifier. The standard deviation (SD) was highest for the neutral diclofenac and minimal for its anionic species. We estimate that partitioning differences between two datasets as

Table 5—Effect of Vesicle and Lipid Types and Analytical Techniques on Partitioning

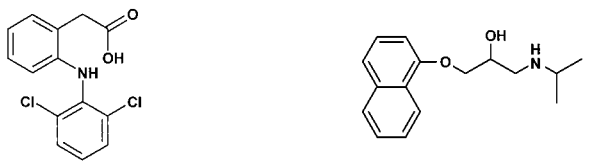
vesicle[a]	size (nm)	lipid	detection	T (°C)	diclofenac		propranolol	
					log $P_{neutral}$	log P_{ion}	log $P_{neutral}$	log P_{ion}
S–SUV	32	Soy-PC	pH-metric	25	4.5	3.0	3.4	2.6
S–SUV	32	Soy-PC	pH-metric	37	4.3	2.9	3.2	2.5
S–SUV	25	DOPC	pH-metric	25	4.3	2.2	3.4	2.1
FAT-LUV	–	Soy-PC	pH-metric	25	4.4	3.3	3.5	2.8
FAT-LUV[7]	100	DOPC	pH-metric	25	4.5	2.6	3.5	2.6
ED-SUV[5]	70	Egg-PC	radiotracer	37	–	–	3.2	2.8
MLV[17]	–	Egg-PC	centrifugation, UV	37	–	–	–	2.4
–	–	octanol	pH-metric	25	4.5	0.7	3.5	0.8

[a] MLV: multilamellar vesicles.

Table 6—Reproducibility

lab	operator	diclofenac		propranolol	
		log $P_{neutral}$	log P_{ion}	log $P_{neutral}$	log P_{ion}
1	1	4.52	3.00	3.40	2.60
2	2	4.49	2.96	3.32	2.53
2	3	4.19	2.94	3.17	2.41
mean		4.40	2.97	3.30	2.51
SD[a]		0.18	0.03	0.12	0.10

[a] SD: standard deviation.



Diclofenac
pK_a (25°C): 3.99
pK_a (37°C): 4.01

Propranolol
pK_a (25°C): 9.53
pK_a (37°C): 9.14

Figure 1—Structure and pK_a of diclofenac and propranolol.

calculated from four titrations at different lipid concentrations may not be significant if they do not exceed ±0.3 log units.

Comparison with Available Partitioning Data—The results obtained with the S-SUV in potentiometric titrations were in good agreement with literature results of membrane partitioning. Equilibrium dialysis results for propranolol liposome partitioning were used as the reference of the first choice. Good agreement could be achieved at 37 °C without any extra equilibrium time (see lines 2 and 6, Table 5). For these experimental conditions, the same partition coefficient was determined for the neutral species of propranolol. The partition coefficient of the cation determined by titration was 0.3 log units lower compared to the equilibrium dialysis result which may not be significant. In ref 16, partitioning results were given for pH 7.4 where propranolol is almost completely present in the cationic form. In the octanol–water partitioning system, drastically smaller partition coefficients have been determined for the ionic species of diclofenac and propranolol.

Conclusions

The use of sonicated small unilamellar vesicles made of soybean-PC as a biomembrane partition model in a pH-metric titration assay was studied with various assay settings. The titrimetric results for the partitioning of propranolol to sonicated small vesicles were in good agreement with results generated with an equilibrium dialysis/radiotracer method at a high lipid-to-drug ratio. Titrimetric partition results measured with S-SUV were also comparable to those obtained with large unilamellar vesicles made by an extrusion technique.

Small unilamellar vesicles made by sonication combine several advantages for its use as a rapid partitioning assay. The production process allows testing at lipid concentrations as high as 100 mg/mL. Therefore partitioning assays can be run at high lipid-to-drug ratios to avoid saturation of lipid with drug. They can also be prepared with simple lab equipment in a short time. Furthermore, partitioning to small vesicles of soybean-PC differed only slightly for minimally from large vesicles despite their more pronounced curvature. The use of pure DOPC instead of soybean-PC did not lead to more relevant partitioning results or other advantages. Since soybean-PC consists of a mixture of acyl-glycerophosphocholines, it should be closer to the composition of biological membranes than pure DOPC. On the basis of the results discussed above, partitioning data to small unilamellar soybean-PC vesicles can be utilized as a potential membrane model and may have advantages over other vesicles. Higher possible lipid concentrations, simple preparation, and low cost of phospholipid make this method attractive as a standard screening procedure. Data generated by this titrimetric assay have been successfully utilized for a correlation of intestinal drug absorption with dose, solubility, and liposome partitioning.[16]

Glossary

S-SUV	Small unilamellar vesicles generated by sonication
ED-SUV	Small unilamellar vesicles generated by equilibrium dialysis
FAT-LUV	Large unilamellar vesicles generated by equilibrium dialysis
Soy-PC	Soybean phosphatidylcholine, purified
MLV	Multilamellar vesicles
DOPC	Dioleoylphosphatidylcholine

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