Biochemical Pharmacology, Vol. 29, pp. 2361–2365.
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0006-2952/80/0901-2361 $02.00/0

PARTITIONING AND EFFLUX OF PHENOTHIAZINES FROM LIPOSOMES

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(*Received* 1 *December* 1979; *accepted* 11 *April* 1980)

Abstract—The partitioning and efflux of eight phenothiazine derivatives from egg phosphatidylcholine liposomes has been studied and a linear relationship between the equilibrium partition coefficient (K) and first-order efflux rate constant derived. Higher coefficients were found for the phenothiazines partitioning into egg phosphatidylcholine compared with dipalmitoylphosphatidylcholine liposomes at 37°. The concentration dependence of K for chlorpromazine was determined and found to increase at low concentrations but to decrease above 1.4×10^{-4}M. The equilibrium partition coefficients of perphenazine were pH dependent, with maximum liposomal incorporation at pH values greater than the pK_a of the base. The efflux of mequitazine was enchanced from liposomes containing cholesterol.

Multilamellar liposomes can entrap ions and small molecules, and release them at controlled and varying rates. This concept has been employed, for example, in cancer chemotherapy [1] and in the treatment of leishmaniasis [2]. Thus it is important to understand the factors governing the distribution and interaction of drug molecules with liposomes.

The distribution of a drug within a liposome depends on its structure and lipophilicity. Drugs are located in either the hydrophobic interior of the bilayer, in the aqueous channels or associated with the polar head groups at the surface of a liposome. Using electron spin-resonance spectroscopy, perphenazine and chlorpromazine have been shown to be preferentially located in the polar part of the bilayer whereas oxidized derivatives of chlorpromazine are found principally in the hydrophobic interior of the bilayer [3]. The measurement of partition coefficients of the drug between the aqueous phase and the pure phospholipid bilayer provides a useful guide to the transport of drugs across the liposome membrane [4]. Although most lipid-soluble ions are not normally absorbed, the transport of some lipid-soluble ions has been reported [5–6] to occur in three steps: (a) adsorption at the membrane interface, (b) diffusion within the membrane, (c) desorption into the aqueous phase from the opposite interface. A number of mechanisms have been implicated in the way in which drugs can partition into liposomes and be transported across the bilayer [7–8].

In this paper, an attempt is made to correlate the partitioning of phenothiazines with transport rates across liposome membranes. The effect of cholesterol, pH, and drug concentration on partitioning and permeation across liposome membranes is also considered. The ability of phenothiazines to alter the permeability of liposomes has been previously studied [9], and the interaction of chlorpromazine with liposomes determined [10, 11]. An extensive study of the partitioning of phenothiazines into dipalmitoyllecithin was recently reported by Jain and Wu [12].

MATERIALS AND METHODS

Materials. The following companies kindly donated the phenothiazine drug samples. May & Baker, Dagenham, Essex, U.K., provided promethazine hydrochloride, trimeprazine tartrate, prochlorperazine mesylate, and chlorpromazine hydrochloride. Wyeth Laboratories, Maidenhead, Berks, U.K., provided promazine hydrochloride. Squibb & Sons, Merseyside, U.K., provided fluphenazine hydrochloride. Allen & Hanburys, Bethnal Green, London, U.K., provided perphenazine base. Berk Pharmaceuticals, Guildford, Surrey, U.K., provided mequitazine base. All the phenothiazine derivatives were of pharmaceutical grade and used without further purification. Egg phosphatidylcholine (EPC) was purified and re-crystallized from the crude egg lecithin (BDH Chemicals) [13]. The purified EPC was stored under acetone at 4°. The re-crystallized EPC was found to be chromatographically pure with an R_f value of 0.55 using chloroform : methanol : H_2O, 14 : 6 : 1 as a solvent system. L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-distearoylphosphatidylcholine (DSPC) (Sigma Chemical Co., St. Louis, MO), and cholesterol (Fluka, A.G.) were used without further purification.

Methods. The preparation of multilamellar liposomes has been the subject of a number of publications (e.g. Refs. 14 and 15). In this study the compounds of very low water solubility were added

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to the organic phase, whereas the more water soluble phenothiazine derivatives were added to the aqueous phase (0.9% saline, pH 7.4). A final concentration of 1 $mg \cdot ml^{-1}$ of phospholipid and 0.1 $mg \cdot ml^{-1}$ of phenothiazine drug was obtained. The adjustment of pH of the aqueous phase was achieved by the addition of either 0.1 M hydrochloric acid or sodium hydroxide solution.

The liposomes were stirred for a predetermined equilibrium time of 28 hr at a constant speed in a water bath kept at 37°. The equilibrium partition coefficient was determined by removing aliquots of the equilibrated liposomes and separating these by centrifugation at 70,000 *g* for 1 hr. The supernatant was assayed by u.v. spectrophotometry (Beckman Model 25, U.S.A.) for free phenothiazine content [10, 16]. The amount of phenothiazine drug associated with the liposomes was found by difference.

The first-order efflux rate constant (k) was determined over a 4-hr period by diluting an equilibrated sample ten-fold with 0.9% saline at 37°. Since chlorpromazine is known to be adsorbed by various materials, e.g. PVC tubing [17], care was taken in the choice of the filtration membrane. Amicon UM20 membranes were found to be the most satisfactory. Aliquots were removed every 30 min and filtered under pressure through an ultrafiltration cell using a UM20 membrane [18]. The free drug solution was assayed as before. All solutions containing phenothiazines were protected from light.

RESULTS AND DISCUSSION

The first-order efflux rate constants (k, hr^{-1}) for the eight phenothiazine drugs were calculated using a general equation of a first order reaction:

$$\ln C_t = \ln C_0 - kt, \quad (1)$$

where C_t = concentration of liposome-associated drug at time t; C_0 = concentration of liposome-associated drug at time $t = 0$; and k = the first-order rate constant.

Hence, from the gradients of the ln per cent liposome-associated drug *vs* time graphs, k may be calculated. Initially, two methods of challenging equilibrium and studying efflux rates from liposomes were investigated. Firstly, the equilibrated sample was diluted ten-fold and the efflux rate followed. Secondly, the sample was centrifuged, the supernatant discarded, the pellet of liposomes resuspended, and the efflux of the drug determined. It was found that the efflux rate from the resuspended liposomes was 7 per cent higher than that from the diluted liposomes. This would be expected as the concentration gradient is higher in the latter case. However, the ten-fold dilution method was selected and used throughout the study due to the ease of manipulation and the possible changes in liposome size distribution resulting from resuspending the pellet.

Figure 1 shows a linear relationship between the equilibrium partition coefficient and the first-order rate constant for phenothiazine efflux from liposomes, using the seven commercially available phenothiazines.

The relationship is

$$K = -20.20\,k + 4.49,$$

with correlation coefficient $r = 0.9766$ and probability $P > 0.99$. This suggests that the transport rate of phenothiazines is determined by the lipophilicity of the drug molecule [19]. From the partitioning data in DPPC and EPC liposomes (Fig. 2), the following linear relationship is found:

$\ln K_{DPPC} = 1.80 \ln K_{EPC} - 1.84$, with $r = 0.8672$, $P > 0.99$ ($N = 8$).

This relationship is of the form suggested by Collander [20]. One can conclude, therefore, that although partitioning into membranes is dependent on the membrane lipid composition, it is possible to derive empirical relationships for similarly structured membrane systems. It is also evident from Fig. 2 that phenothiazine drugs partition more into EPC

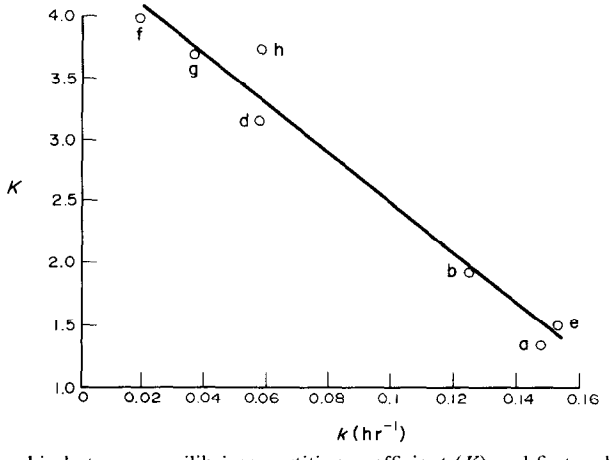

Fig. 1. The relationship between equilibrium partition coefficient (K) and first-order rate constant (k, hr^{-1}) for seven phenothiazine derivatives in egg phosphatidylcholine liposomes. Temperature 37°, pH 7.4. (a) Promethazine hydrochloride, (b) trimeprazine tartrate, (c) prochlorperazine mesylate, (d) chlorpromazine hydrochloride, (e) promazine hydrochloride, (f) fluphenazine hydrochloride, (g) perphenazine base, (h) mequitazine base. The straight line gives the best mean squares fit.

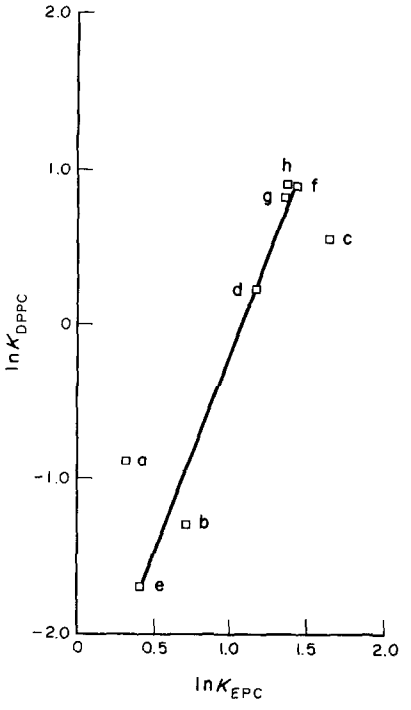

Fig. 2. The relationship between the partitioning of eight phenothiazines in dipalmitoylphosphatidylcholine and in egg phosphatidylcholine liposomes. Temperature 37°, pH 7.4.

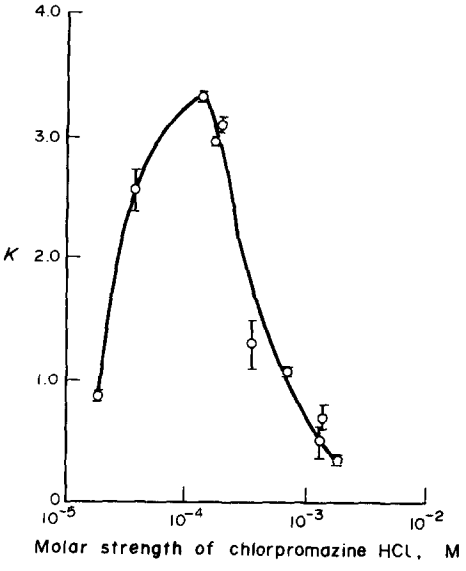

Fig. 3. The relationship between equilibrium partition coefficient (K) and varying concentrations of chlorpromazine HCl in liposomes containing 1.28×10^{-3}M egg phosphatidylcholine. Temperature 37°, pH 7.4. The standard deviation is shown as a vertical bar.

liposomes than into DPPC liposomes. This is to be expected since the EPC liposomes are in a more fluid state at 37° compared with the DPPC which is in the more rigid gel or crystalline state and has a transition temperature of 41°.

Since pH controls the ratio of ionized to unionized drug, it plays an important role in the absorption of drugs from the gastrointestinal tract and their subsequent distribution in the body. Perphenazine, which has a second pK_a of 7.8 [21], was used to study this phenomenon *in vitro*. The partioning of perphenazine was found to increase with increasing pH up to 7.8, the pK_a of the drug. Little increase in partitioning was observed at pH values above the pK_a. For maximum incorporation of perphenazine into lipid bilayers and hence membrane transport, the pH should therefore exceed the pK_a of the base. These results are in agreement with the work of Seeman and Kwant [22] who showed that chlorpromazine-induced membrane expansion in erythrocyte ghosts increased with increasing pH.

The concentration-dependent distribution of chlorpromazine within biomembranes was assessed by measuring the partition coefficient (K) in EPC liposomes (Fig. 3). K was found to increase at low concentrations, but to decrease above 1.4×10^{-4}M, which may reflect pre-micellar aggregation and dimer formation of the chlorpromazine. At post critical micelle concentrations, solubilization of the liposome occurred, possibly caused by the formation of mixed micelles. The formation of pre-micellar aggregates and mixed micelles in the aqueous phase reduced the distribution of chlorpromazine in the phospholipid bilayer. Our results for chlorpromazine are in agreement with the published data [23].

Rigid molecules of cholesterol are a component of natural membranes together with other lipids. In the mitochondria and microsomes of liver the cholesterol : phospholipid ratio ranges from 0.11 to 0.33, whereas mitochondria from brain and intestine are found to have higher cholesterol : phospholipid ratios of 0.51 and 0.6 [24]. The effect of cholesterol on the partitioning of mequitazine into DPPC liposomes was therefore investigated. The incorporation of mequitazine into DPPC liposomes was found to be dependent on the concentration of cholesterol in the liposomes (Fig. 4). The amount incorporated decreased up to a mole ratio of 1.0 and thereafter increased with increasing cholesterol content. It has been suggested that above 1:1 mole ratio cholesterol : phospholipid, the system coexists in two phases, that is, a 1:1 cholesterol : phospholipid complex and free crystalline cholesterol [25]. The interaction of cholesterol with phospholipids has been studied using various instrumental techniques [26] and the maximum molar ratios of lecithin cholesterol in aqueous dispersions reported to be 2:1, 1:1, or 1:2. The evidence suggests the complex 1:1 is metastable.

Table 1 shows the efflux of mequitazine from various phospholipid liposomes in the absence and presence of cholesterol at 2:1 w/w ratio of phospholipid : cholesterol. All four phospholipids retain mequitazine poorly in the presence of cholesterol. The results for DPPC and DSPC liposomes are in accordance with the literature, where it has been suggested that cholesterol enhances the permeability of liposomes derived from saturated lecithins below their transition temperature [24]. This is

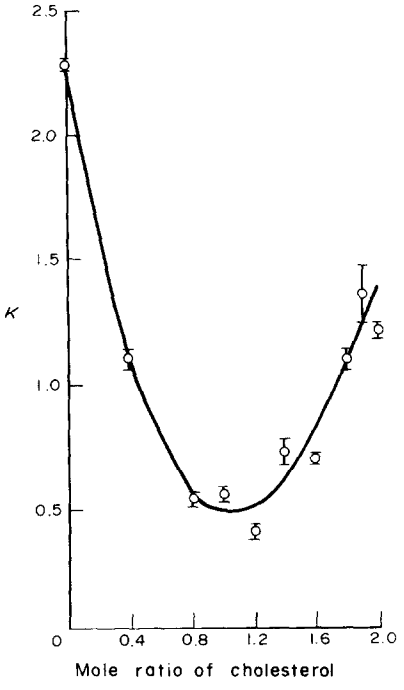

Fig. 4. The effect of cholesterol concentration on the partitioning of mequitazine base between dipalmitoylphosphatidylcholine liposomes and 0.9% w/v saline at 37°, pH 7.4. Equilibrium partition coefficient (K) is calculated on the basis of total lipid present. The standard deviation is shown as a vertical bar.

because cholesterol can exhibit a liquifying effect on crystalline lipids, leading to an intermediate gel state. At temperatures above the transition temperature, cholesterol has a condensing effect on the bilayer and is therefore expected to reduce the permeability of liposomes. However, this was found not to be the case for mequitazine efflux from EPC and DMPC liposomes, with transition temperatures well below the experimental temperature of 37°. For better retention of mequitazine, the liposomes consisting of phospholipids alone would be more appropriate.

Figure 5 shows the effect of the length of saturated hydrocarbon chains in the phospholipid molecule on the efflux of mequitazine in the absence and presence of cholesterol. In the absence of cholesterol, the partitioning and hence the amount of mequitazine incorporated is dependent on the hydrocarbon chain length. The incorporation of mequitazine decreases with increasing chain length. The transport of mequitazine, indicated by the first-order rate constant, is also dependent on the chain length and increases with an increase in chain length. In the presence of cholesterol the partitioning of mequitazine exhibited little dependence on the saturated hydrocarbon chain length whilst the transport was found to increase markedly with chain length. Cholesterol addition resulted in higher values of k and lower values of K. Hence, for the entrapment and release of drugs from liposomes, the absence or presence of cholesterol is an important factor. Elevated cholesterol levels *in vivo* therefore may result in changes in the kinetics of drug absorption.

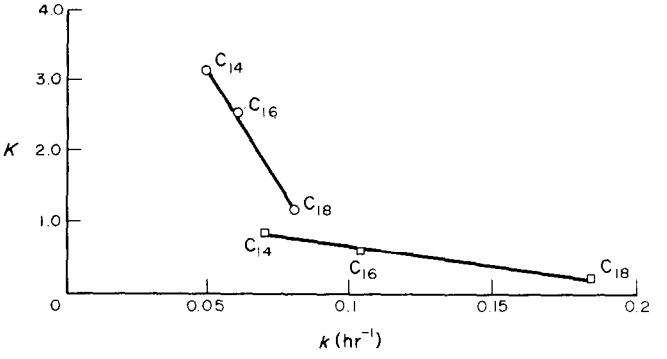

Fig. 5. The effect of phospholipid chain length on the relationship between equilibrium partition coefficient (K) and the first-order rate constant (k, hr^{-1}) of mequitazine base at 37°, pH 7.4. ○, phospholipid alone; □, phospholipid: cholesterol ratio, 2:1 w/w. C_{14}, C_{16}, and C_{18} are dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine, respectively.

Table 1. The release rates of mequitazine base from various phospholipid liposomes. Phospholipid: cholesterol ratio of 2:1 w/w

	First order rate constant (k, hr^{-1})	
Phospholipid in liposomes	Without cholesterol	With cholesterol
Egg phosphatidylcholine	0.0598	0.1824
Dimyristoylphosphatidylcholine	0.0489	0.0704
Dipalmitoylphosphatidylcholine	0.0620	0.1043
Distearoylphosphatidylcholine	0.0786	0.1852

Phenothiazines, being amphiphilic molecules, are useful model drugs for studying distribution, retention and penetration of drugs in liposomes. The information obtained from such physical studies may assist in the interpretation of the kinetics and route of penetration of the drug to its site of action *in vivo*. The thermodynamics of partitioning into liposomes of closely related phenothiazines presently being investigated should permit a group-contribution analysis to be undertaken of the relationships existing between phenothiazine structure and membrane transport.

Acknowledgements—We thank the S.R.C. for a CASE studentship for M.A. and Berk Pharmaceuticals Ltd. for financial support.

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