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A quantitative approach to the determination of drug release from reverse-phase evaporation lipid vesicles. The influence of sodium ion-pair formation on warfarin partitioning and permeability

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Summary

The influence of sodium ion-pair formation on warfarin partitioning and permeability has been investigated using reverse-phase evaporation lipid vesicles. An experimental method for the isolation of the vesicles having known amounts of encapsulated drug has been described. The partitioning of warfarin between phospholipid membrane and aqueous phase at different Na^+ concentrations was determined in separate experiments. Thus, using a two-compartment cylinder assembly the influence of Na^+ concentration on the release rate of the encapsulated warfarin from the vesicles could be measured quantitatively. It appears that under the present experimental conditions warfarin partitioning does depend on Na^+ concentration whereas vesicle permeability towards warfarin does not seem to be affected.

Introduction

Several investigators have studied the efflux of drugs from phospholipid vesicles. Multilamellar (Ahmed et al., 1980; Arrowsmith et al., 1983) and unilamellar (Hermetter and Paltauf, 1981; Muranushi et al., 1980; Juliano and Stamp, 1979) systems have been examined from two main points of interest. First, phospholipid

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bilayers may simulate some fundamental properties of biological membranes and constitute a model system to investigate passive drug transport. Second, there is the possible application of phospholipid vesicles as a drug delivery system. Both objectives have been faced with the serious problem that until recently no method for the preparation of a homogeneous and well-characterized population of unilamellar vesicles with a sufficiently large internal volume was available. Yet, knowledge of these properties is necessary for the purpose of a quantitative approach of transport phenomena if water-soluble compounds are to be encapsulated and released. Fortunately a method for the preparation of phospholipid vesicles that would meet those requirements was described by Szoka and Papahadjopoulos (1978). We used this method in our present investigation where we studied the influence of ion-pair formation on the efflux rate of the anticoagulant warfarin from phospholipid vesicles.

In a previous communication from our laboratory (van der Giesen and Janssen, 1982) it was reported that the apparent partition coefficient of warfarin at pH 10.0 (warfarin has a pK of 5.0 and is completely dissociated under those conditions) was increased by the presence of Na^+ ions. A linear relationship between the logarithm of the apparent partition coefficient K_{app} and the negative logarithm of the sodium ion concentration pNa was observed. This result could be explained by assuming the formation of ion-pairs between the anion of warfarin and Na^+. In a follow-up study we found a linear relationship between the logarithm of the rate constant for the diffusion of warfarin through octanol-impregnated Millipore filters and pNa (Cools and Janssen, 1983).

It seemed appropriate to extend the investigations on the influence of ion-pair formation on transport kinetics to the area of phospholipid bilayer passage, to find out if comparable results could be obtained in a more realistic but at the same time more complicated model system.

Materials and Methods

Chemicals

Warfarin was purchased from ACF Chemiefarma (ACF, Maarssen, The Netherlands) and used as supplied. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Fluka, Sephadex G25 coarse from Pharmacia and Triton X-100 from Sigma Chemicals. All experiments were performed at pH 11.5 (10 mM NaOH).

Measurement of the partitioning of warfarin between phospholipid membrane and aqueous phase at different Na^+ concentrations

DPPC (40 mg) was dissolved in 5 ml chloroform in a round-bottomed flask and dried in vacuo in a rotary evaporator. The remaining phospholipid film was suspended in 5.5 ml solution, containing warfarin (0.05 mM), NaOH (10 mM) and NaCl (0 mM, 40 mM, 100 mM, 500 mM, 1000 mM). The multilamellar liposomes formed in this way, essentially as described by Bangham et al. (1965), were stored at 45 °C for 1 h and thereafter at 30 °C for the next 20 h before use. Liposomes (5 ml)

were then transferred to a polypropylene centrifuge tube. 6 centrifuge tubes, containing liposomes formed at different Na^+ concentrations, were centrifuged in an ultracentrifuge (MSE superspeed 75) at 100.000 g for one night at 30°C. The next day the warfarin concentrations of the supernatants were measured. It was determined that no warfarin was adsorbed to the walls of the centrifuge tubes.

For the purpose of the determination of the apparent partition coefficients (K_{app}) the membranous volume of the liposomes in the gel state was calculated, taking into account a value of 45 $Å^2$ for the cross-sectional area of one DPPC molecule and a value of 42 Å for the thickness of a bilayer. The values are taken from Chapman et al. (1967) and from Levine et al. (1973). 1 mg DPPC then corresponds to a membranous volume of 0.776 μl.

Preparation of reverse-phase evaporation lipid vesicles

Reverse-phase evaporation lipid vesicles (REVs) were prepared at different Na^+ concentrations according to the method of Szoka and Papahadjopoulos (1978). 20 mg DPPC was dissolved in 5 ml ether and 5 ml chloroform in a round-bottomed flask. 1.5 ml Solution containing warfarin (6 mM), NaOH (10 mM) and NaCl (0 mM, 30 mM, 100 mM, 600 mM, 1000 mM) was added next and the mixture treated ultrasonically at 50°C in an ultrasonic bath for 3 min. At the end of that time a milky single-phase dispersion was left. Then the ether and chloroform were carefully evaporated at reduced pressure at 45°C from the round-bottomed flask which was fitted to a rotary evaporator by means of a long extension glass tube. The conditions were such that the volume of a REV preparation after complete evaporation of the organic liquids amounted to about 1.1 ml. The REVs were stored at 45°C for one hour and at 30°C for the next 20 h before use.

The final volume of a preparation will hereafter be called V. Warfarin, NaCl and DPPC then will be concentrated by a factor $1.5\ V^{-1}$. Two portions of 0.5 ml of each preparation were used separately as described below. At this point it should be noted that each portion will contain $20 \times 0.5 \times V^{-1}$ mg DPPC corresponding with a membranous volume V_M:

$$V_M = 20 \times 0.5 \times V^{-1} \times 0.776 \times 10^{-3}\ (\text{ml}) \tag{1}$$

Subtracting V_M from the total volume of a portion (0.5 ml) then yields the volume of the aqueous phase V_A:

$$V_A = 0.5 - V_M\ (\text{ml}) \tag{2}$$

The total amount of warfarin, m_{tot} (μmol), in each portion may be derived from the original warfarin concentration. The concentration of warfarin in the aqueous phase, $[W]_A$, may be expressed either as the quotient of the amount of warfarin in the aqueous phase and the volume of the aqueous phase, or as the concentration of warfarin in the membranous phase divided by K_{app} (Eqns. 3 and 4):

$$[W]_A = \frac{m_{tot} - m_M}{0.5 - V_M}\ (\text{mM}) \tag{3}$$

$$[W]_A = \frac{m_M}{K_{app} \cdot V_M} (mM) \quad (4)$$

Equating the last parts of expressions 3 and 4 yields the value for the amount of warfarin in the membranous phase:

$$m_M = \frac{K_{app} \cdot V_M \cdot m_{tot}}{K_{app} - V_M + 0.5 - V_M} (\mu mol) \quad (5)$$

It then follows that the amount of warfarin in the aqueous phase is:

$$m_A = m_{tot} - m_M (\mu mol) \quad (6)$$

Isolation of REVs using gel filtration

To remove the warfarin not captured by the vesicles 0.5 ml REVs were put on top of a Sephadex column (G25 Coarse, 10 ml bed volume, diameter 10 mm) and eluted with the same solution as used for the preparation of the vesicles except that warfarin was absent (elution rate 0.9 ml/min). This solution also was used to swell the Sephadex before use. The elution procedure was performed twice in exactly the same way for each vesicle preparation. The first time it was used to characterize the elution pattern at that specific Na^+ concentration and to determine the amount of warfarin encapsulated in the REVs (m_{REV}). Therefore subsequent fractions of 0.6 ml were collected and diluted with water to 12.8 ml. The samples were treated with Triton X-100 (1%) at 60 °C for 2 h to lyze the vesicles. Then warfarin was measured at 308 nm.

The elution patterns showed two peaks: a first narrow peak of warfarin that had been captured by the REVs and a second broad one containing bulk warfarin. The amount of warfarin encapsulated in the REVs, m_{REV} (μmol), was derived from the sum of the absorbances of the first peak using 13.6 as the molar extinction coefficient of warfarin. The amount of warfarin in the internal water phase (m_I) then could be found by subtracting the amount of warfarin in the membranous phase from m_{REV}:

$$m_I = m_{REV} - m_M (\mu mol) \quad (7)$$

The volume of the internal aqueous phase then was given by:

$$V_I = \frac{m_I}{[W]_A} (\mu l) \quad (8)$$

The result of the elution patterns was used to predict the warfarin content of a specific amount of REVs (2.0 ml, i.e. 3.4 fractions) which in an analogously performed second elution, were collected at once for the purpose of an efflux experiment. The quotient of the warfarin content of that specific amount of REVs

and the warfarin content of all the REVs eluted in the first elution was used to find the values of m_I, m_M, V_I and V_M for that specific amount of REVs.

A single isolation procedure could be performed within 8 min.

Measurement of diffusion of warfarin out of REVs

For this purpose a slightly modified cylinder assembly was used as described before (Cools and Janssen, 1983). The volume of the two compartments this time was 2.3 ml and 17.0 ml, respectively. Into the smaller barrel 2 ml isolated REVs were pipetted. A Millipore filter (type VC, pore size 0.1 μm) was applied to the top of this barrel. The pore size was small enough to prevent permeation of vesicles through the filter, whereas warfarin could pass through it easily (see below). This membrane therefore functions as a separating or dialysis membrane. In the text this membrane will be referred to as the dialysis membrane in order to avoid confusion with the phospholipid membranes.

The bigger barrel was filled with 15 ml of the same solution that was used to eluate the REVs from the Sephadex column. A light cell was placed on top of this barrel. The system actually is assumed to be built up of 4 compartments. The REVs in the smaller barrel make 3 compartments: an internal aqueous phase (compartment I), a membranous phospholipid phase (compartment M) and an external aqueous phase (donor compartment D). The solution in the larger barrel makes a second external aqueous phase (receptor compartment R).

At the beginning of an efflux experiment compartments I and M contain warfarin, whereas compartments D and R do not. The assembly was turning around at 60 Hz on a roller mixer at 30 °C. Warfarin was measured at 308 nm in a thermostated cuvet-holder, at 30 °C at fixed time intervals.

Measurement of the diffusion of warfarin through the Millipore filter in the absence of vesicles

The smaller barrel was filled with 2 ml warfarin (0.4 mM), NaOH (10 mM) and NaCl (0 mM, 30 mM, 100 mM, 600 mM, 1000 mM). As before a Millipore filter was applied on top of this barrel. Then the larger barrel was filled with 15 ml of the same solution except that warfarin was absent. A light cell was applied to the top of this barrel and the assembly was placed on the roller mixer under the same conditions as pointed out before. Warfarin again was measured at 308 nm, at fixed time intervals.

Results and Discussion

Influence of Na^+ concentration on the partitioning of warfarin between phospholipid and aqueous phase

A linear relationship (Eqn. 9) between log K_{app} and the negative logarithm of the sodium ion concentration represented as pNa was found over the whole Na^+ concentration range investigated.

$$\log K_{app} = -0.30\ (\pm 0.02)\ pNa + 1.02\ (\pm 0.02) \qquad (9)$$

$(n = 5, r = 0.995, s = 0.03)$

This result was used to calculate the apparent partition coefficient of warfarin between the internal aqueous phase and the phospholipid membrane phase of the vesicles at the beginning of an efflux experiment.

The pNa values used in our experiments are all between 0.00 and 2.00. This implies that log K_{app} varies between 0.4 and 1.0 according to Eqn. 9. This change is much smaller than was observed earlier by van der Giesen and Janssen (1982) using octanol as the organic phase. They found the coefficient of pNa to be -1.00 in comparable experiments in the pNa range of -0.4 to 1.2. Later experiments covering a wider range, showed that this coefficient in fact was near -0.6 (van der Giesen, unpublished experiments). This different value of the coefficient of pNa demonstrates the effect of the organic phase on the partition behavior of sodium warfarin ion-pairs.

The effect of the character of the organic phase on partition coefficients is well documented and mostly described by a Collander-type equation (Leo, 1972). From the results mentioned above a relationship between partition coefficients in DPPC and octanol can easily be derived for sodium warfarin ion-pairs. This relation will take the form:

$$\log K_{app}(\text{DPPC}) = a \log K_{app}(\text{octanol}) + b \qquad (10)$$

where a and b are constants. A value between 0.3 and 0.5 for a has to be expected. This means that the discriminative power of DPPC is much smaller than that of octanol. This is in accord with recently reported results (Arrowsmith et al., 1983) concerning the partitioning of aliphatic cortisone-21 esters in DPPC liposomes. They reported that the incremental free energy of partitioning per methylene group was strongly dependent on chain length. Its value appears to be much lower than the value of -3.0 kJ per CH_2 (in octanol), calculated from the hydrophobic fragmental constant value of 0.53 (Rekker and de Kort, 1979) using the method described earlier (Janssen and Perrin, 1976).

Further it should be noted that the experiments in the present investigation were performed at 30°C, i.e. about 11°C below the phase transition temperature of DPPC. The partitioning of solutes between phospholipid bilayers and water has been reported to depend on temperature and especially on the phase, either gel or liquid crystalline phase, of the bilayers (Ahmed et al., 1981; Melchior and Steim, 1976). Partition coefficients seem to be lower in the case of bilayers in the gel phase. Probably the more tight chain packing in bilayers in the gel phase is responsible for the reduction of partition coefficients under those conditions (Cohen, 1975). A straight comparison with organic liquid and water systems is hampered because phospholipid bilayers do not constitute a homogeneous organic phase. The inner core consists of long-chain phospholipid tails that represent a more lipophilic region whereas the outer core consists of polar phospholipid head groups that represent a more hydrophilic region.

Determination of diffusion rate constant k_d through the dialysis membrane

The diffusion of warfarin through the Millipore membrane used to separate the

TABLE 1

INFLUENCE OF Na^+ CONCENTRATION ON WARFARIN TRANSPORT

pNa	$k_d(10^{-3}\ min^{-1})$	$k_r(10^{-3}\ min^{-1})$	$P(10^{-6}\ cm \cdot min^{-1})$
1.87	20.5	1.4	9.7
1.23	20.8	1.7	9.8
0.84	21.5	1.8	7.0
0.10	20.9	4.6	13.1
−0.11	20.0	4.0	11.1

pNa = negative logarithm of Na^+ concentration; k_d = Millipore filter diffusion transport constant of warfarin determined in absence of REVs according to Eqn. 11; k_r = release rate constant of warfarin from REVs according to Eqn. 18 (mean standard deviation = 0.10×10^{-3}); P = permeability constant for the release of warfarin from REVs according to Eqn. 19.

vesicles from the receptor compartment will obey reversible kinetics. The diffusion rate was measured in the absence of vesicles. Eqn. 11 describes this process:

$$\ln\left(1 - \frac{C_{R,t}}{C_{D,o}} \cdot \frac{V_R + V_D}{V_D}\right) = -k_d t \tag{11}$$

(Albery et al., 1976; Guy and Hadgraft, 1981) where k_d is the diffusion transport constant, $C_{R,t}$ is the concentration of the diffusing warfarin in the receptor compartment R at time t, $C_{D,o}$ is the concentration of warfarin in the donor compartment D at time zero. V_R and V_D represent the volumes of the R and D compartment. In Eqn. 11 it is assumed that $C_{R,t}$ is zero at the start of an experiment. It should be noted that the diffusion transport constant k_d is a function of V_R and V_D as well

$$k_d = k_d^*\left(V_D^{-1} + V_R^{-1}\right) \tag{12}$$

In our experiments $C_{R,t}$ is the quantity which is measured spectrophotometrically. When the results are analyzed according to Eqn. 11, the k_d values as reported in Table 1 are obtained. The average value is 0.0208 min^{-1}, which corresponds with a $t_{1/2}$ (defined as the time when the concentration in the receptor compartment has reached half of the final value) of 34 min.

It is obvious that k_d does not depend on Na^+ concentration. This indeed has to be expected because in this situation the diffusion through aqueous channels in the dialysis membrane will be rate-limiting. Hereafter the average value of k_d will be used as a Na^+ concentration-independent constant.

Influence of Na^+ concentration on elution pattern

The isolation procedure described in the section Materials and Methods proved to be very reliable and effective. The total amount of warfarin eluted from the Sephadex column, i.e. the sum of the amount of warfarin encapsulated in the REVs (first peak) and the amount of free warfarin (second peak) was always almost equal to the total amount of warfarin used for the preparation of the REVs. The first 2 ml

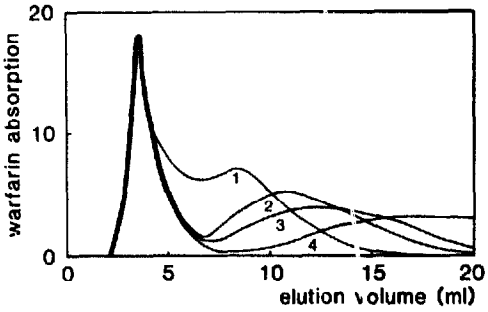

Fig. 1. Isolation of warfarin containing REVs. Warfarin absorption at 308 nm of subsequent fractions of a REV preparation eluted from Sephadex columns. pNa of REV preparations: 1 = 1.87; 2 = 1.23; 3 = 0.84; 4 = −0.11.

of vesicles dropping from the column were not contaminated with bulk warfarin at any Na^+ concentration as can be seen from Fig. 1. When the vesicles were rechromatographed the bulk free warfarin peak had completely disappeared. The isolation procedure, therefore, is suited for the purpose of quick and effective separation. Table 2 gives a summary of the composition of the REV preparations used in the experiments. The internal volume of the REVs at the lowest Na^+ concentration used amounts to 11.8 μl/mg phospholipid. Furthermore there appears to be a gradual decrease of the internal volume when the Na^+ concentration is raised. Both observations are in good agreement with the findings of Szoka and Papahadjopoulos (1978).

Release of warfarin from REVs and analysis of release experiments

The release of warfarin was followed by measuring the increase in absorbance in the receptor compartment over at least 5 h. It appeared that about 25% of the total warfarin encapsulated in the REVs was released during this time.

The results were analyzed according to the model described by Chowhan et al. (1972). In this model it is assumed that the drug instantaneously partitions between phospholipid and internal aqueous phase and that the only barrier to transport from

TABLE 2

ENCAPSULATION PARAMETERS OF VESICLE PREPARATIONS

pNa	K_{app}	m_I(μmol)	m_M(μmol)	V_I(μl)	V_M(μl)
1.87	2.9	0.58	0.11	72	4.7
1.23	4.5	0.60	0.21	72	5.6
0.84	5.9	0.32	0.23	46	5.6
0.10	9.8	0.17	0.28	25	4.3
0.11	11.2	0.20	0.39	30	5.3

pNa = negative logarithm of the Na^+ concentration; K_{app} = apparent partition coefficient of warfarin in the system DPPC–water. Note that K_{app} values in this table are calculated from Eqn. 9; m_I = amount of warfarin inside the REVs at t = 0; m_M = amount of warfarin in phospholipid membrane at t = 0. V_I, V_M = internal and membrane volume of REV's. For further details see text.

the vesicles is located at the external lipid–aqueous interface. The release of warfarin from our REVs then, according to the model, may be described by Eqn. 13:

$$-\frac{dC_A}{dt} = k_r(C_I - C_D) \tag{13}$$

where C_A is the average concentration of warfarin on the inside of the barrier, C_I is the concentration in the internal aqueous phase of the vesicles, C_D is the concentration in the donor compartment D and k_r is a velocity constant. It can be calculated that during the course of an efflux experiment $C_D \ll C_I$. With this assumption Eqn. 13 may be written as:

$$-\frac{dC_A}{dt} = k_r C_I \tag{14}$$

Chowhan et al. obtained irreversible transport kinetics from the donor compartment to the receptor compartment by keeping the concentration in the receptor compartment zero (sink conditions). However, in our experimental approach, we had to take into consideration the back transport from the receptor compartment R to the donor compartment D. The rate expression for warfarin concentration in compartment D then will be given by:

$$\frac{dC_D}{dt} = -\frac{V_I + V_M}{V_D} \cdot \frac{dC_A}{dt} - \frac{V_R}{V_D + V_R} \cdot k_d \cdot (C_D - C_R) \tag{15}$$

The rate expression for warfarin concentration in compartment R finally is given by:

$$\frac{dC_R}{dt} = \frac{V_D}{V_D + V_R} \cdot k_d \cdot (C_D - C_R) \tag{16}$$

The solution of Eqns. 14–16 results in:

$$C_{R,t} = \frac{m_I + m_M}{V_D + V_R} \cdot \left[1 - \frac{1}{k_d - k_r^*}\left(k_d e^{-k_r^* t} - k_r^* e^{-k_d t}\right)\right] \tag{17}$$

where

$$k_r^* = \frac{V_I + V_M}{V_I + K_{app} V_M} \cdot k_r \tag{18}$$

In Eqn. 17, where the warfarin concentration in the receptor compartment $C_{R,t}$ is expressed as a function of time, all constants are known except for the velocity constant k_r^*. By substituting our efflux data into this equation we could derive the best fitting values for k_r^* and k_r at different Na^+ concentrations by means of a non-linear least-squares program. These values are shown in Table 1. The release

rate of warfarin expressed in terms of the velocity constant k_r has increased by about a factor 2.5 at the higher Na^+ concentrations. However, the physical meaning of this increase is restricted because k_r will depend on total internal volume and total membrane surface. As the internal volume (V_I) is decreasing at higher Na^+ concentration (see Table 2) and as total membrane surfaces will depend on the total amount of lipid present, the values of k_r should be corrected to get true permeabilities. Therefore the results finally were expressed in terms of permeability coefficients P:

$$P = \frac{k_r V_I}{S_M} \tag{19}$$

where S_M represents the total membrane surface of vesicles in a specific experiment. S_M was calculated as the quotient of total membrane volume (V_M) and membrane thickness (42 Å). The permeability coefficients P at different Na^+ concentrations are shown in Table 1. It now appears that all permeabilities are of the same order of magnitude and, notwithstanding the numerous experimental manipulations, almost equal. Therefore it may be inferred from this final result that if there should be an influence of Na^+ concentration on permeability then it certainly is a very small effect.

Concluding Remarks

Recently Arrowsmith et al. (1983) investigated the release of aliphatic cortisone-21 esters from DPPC liposomes. From their experiments these authors concluded that the less hydrophobic steroids are leaking faster from liposomes. This phenomenon, which is opposite to what might be expected, was explained by the assumption of steroid retention in the phospholipid bilayers. Because Arrowsmith et al. studied the efflux of highly hydrophobic compounds from multilamellar liposomes, which have a comparatively small internal water–phospholipid ratio, only a very small fraction of the diffusing solute will be present in the internal aqueous compartments of the liposomes. It should be realized that under such conditions mainly the efflux from the phospholipid membranes is studied. This same remark holds for the experiments of Ahmed et al. (1980) concerning the efflux of phenothiazines from multilamellar liposomes. In our experiments the objective was to investigate drug transport from the inside of vesicles across the phospholipid membranes to the outside. For this purpose we used REVs which have a relatively high water–phospholipid ratio.

From our results we have to conclude that warfarin permeability of REVs under the documented conditions does not clearly depend on Na^+ concentration, whereas warfarin partitioning between phospholipid membrane phase and aqueous phase does depend indisputably on Na^+ concentration. This is in contrast with the results of our previous study (Cools and Janssen, 1983) where we investigated the same subject using octanol-impregnated filters instead of the more complicated vesicle membrane system in the present study. The difference in outcome may be explained

in several ways. Of all possibilities we wish to mention a few. For example, it cannot be excluded that the diffusion from the internal aqueous phase of a vesicle to the phospholipid membrane is the rate-limiting step due to the formation of an unstirred diffusion layer as a consequence of insufficient mixing on that side of the vesicle membrane. Another possibility is that the increase in ionic strength at higher K_{app} values introduces an artefactual effect on permeability characteristics. Also it is possible that the actual variation of K_{app} values is too small to allow a detectable influence on the permeability in the vesicle membrane approach. To obtain more insight in the validity of the documented method it now seems appropriate to test a series of compounds covering a comprehensive range of lipophilicity without the inevitability of simultaneous changes in ionic strength.

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