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Quantitative effects of interacting variables on vitamin K_1, phylloquinone, entrapment in liposomes

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Summary
Vitamin K_1 is poorly absorbed by the oral route in certain diseased conditions. Entrapment of this vitamin in liposomes is expected to improve oral absorption. In this study, vitamin K_1 has been encapsulated into multilamellar vesicles (MLVs) composed of either egg phosphatidylcholine (egg PC, EPC) or dipalmitoyllecithin (DPPC) by the classical film method involving hydration of lipids by either hand shaking or vigorous vortex mixing. Amounts of vitamin K_1 ranging from 4.4 to 22.1 μMol were employed to medicate liposome suspensions (26μMm or 13.6μM). A 2^3 factorial design was employed to quantitative the effects of the interaction of certain variables *viz:* nature of lipid (L), method of agitation (M) and initial drug concentration (C), on encapsulation capacity (EC) of liposomes. The results of this study suggest, in particular, that the concentration of the drug (C) and the nature of the lipid-drug interactions (L-C) are the most influential variables. This probably infers that any pre-formulation studies with this drug delivery system should start with a consideration of L-C interaction. A negative eorrelation was found between initial drug concentration and encapsulation capacity of liposomes.

Keywords: *Vitamin K_1 liposomes, encapsulation efficiency, interacting variables, quantitative effects.*

Résumé
La vitamine K_1 est tres pen absorbee par voie orale dans certaines conditions de maladie. d'arret de cette vitamin dan les liposomes est ceuse augmenter l'absorption orale. Dans cette itude, la vitamin K_1 a ete incaplulee en vesicules multilamelaires (VMLs) · composees soit d'ent phosphatidylchiline (oenf PC, EPC) on dipalmitoyllecitine (DPPC) par la methode de film classique associant l'hydratation des lipides soit par la salutation a la main on le mixage vigoureux du vortex. Des quantities de vitmain k1 allount de 4,4 a 22, 1μ mol out ete employees pour remedier a la suspension des liposomes (26μ m on 13,6μ m). Une conception factorielles de 2^3 a ete employees pour quantifier les effects de l'interaction de certaines variables viz la nuture des lipides (L), la methode d'agitation (M) et lu concentration initiale du midicament (C), la capaciti d' encapsulation des liposomes (EC). Les resultats de cette etude suggerent, en particulier que la concentration du mideicament (C) et la nature de l'interaction lipide-medicament (L-C) sont les variables les plus influenctes. Ceci probablement monte que tonte etude pre-formulatoire a vec ce systeme d'administration du medicament devrait commencer par une prise en consideration de l'interaction L-C. Une correlation negative a ete obtenue entre la concentration initiale du medicament et lu, capacite encapsulation des liposomes.

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Introduction

The unique feature that makes liposomes suitable as drug delivery system is their ability to encapsulate a variety of drugs in the phospholipid bilayer structures [1,2].

Since pharmacological effects are often correlated with the bioavailable dose at the target, a knowledge of the absolute quantity of material entrapped inside liposomes may be of predictive value in formulation manipulation for both *in-vitro* and *in-vivo* studies. The bilayer composition is thought to influence the physico-chemical behavior of the formed liposomes, such as encapsulation ability and size [3,4]. Verumi and Rhodes [5] have also reported the influence of altering process conditions on drug encapsulation. They posited that the method of hydration of polar head group probably has a strong influence on the encapsulation capacity of liposomes.

Several methods are now available that can be employed to optimize the amount of drugs encapsulated in vesicles [6]. Although the relationship between formulation and /or process variables and properties of traditional dosage forms have been demonstrated [7-8], not much has been done to quantify the effects of these variables in liposomal drug delivery design, knowledge of which would be valuable in pre-formulation studies.

This study is, therefore, aimed at systematically examining, in detail, the quantitative effects of some formulation variables such as bilayer membrane composition, initial drug concentration and process variable (hydration method) and their possible interactive effects on the encapsulation capacity of liposomes. Intermolecular interaction would modify the hydrocarbon chain of the phospholipids and hence affect the integrity of formed liposomes. A lipid soluble drug, vitamin K_1 (phylloquinone) was selected as a model drug for this study. It has been shown previously that co-evaporation of lipids and lipophilic compounds from organic solvents generates practically good incorporation results [9-10], a finding which has been exploited in this study.

Experimental design

The nature (level of saturation) of lipid (L), method of hydration of dried lipid film in aqueous buffer (M) which determines lamellarity, size and amount of entrapped volume as well as the concentration of free drug added, (C), were studied for their effects on encapsulation capacity of liposomes (EC). To quantitate the effects of L, M and C on EC, each of these variables was employed at a 'high' level (denoted by the subscript, H) and a 'low' level (subscript, L) in a 2^3 factorial experimental design as shown in Table I [11]. Thus the eight possible combinations are as follows:

$L_L M_L C_L$; $I_L M_L C_H$; $L_L M_H C_H$; $L_H M_L C_L$;
$L_L M_H C_L$; $L_H M_L C_H$; $L_H M_H C_H$; $L_H M_H C_L$.

To assess the effect that a variable had separately on EC and to determine whether the variable was acting independently or interacting, the following treatments were applied:

Table 1: Mean sizes of liposomal formulations studied

Liposome formulation	Mean sizes (μm) ± S.D
L_HM_L	2.5 ±1.6
L_LM_L	3.0 ±1.6
L_LM_H	2.3 ±1.1
L_HM_H	2.0 ±1.0

Key: *Subscripts 'H' and 'L' denote high and low levels of variable C, M and L*

(i) independent effects, e.g., effects of changing L from its 'low' level (L_L) to its high'level (L_H)

$$L_{n-1\,(L \rightarrow H)} = Y_H - Y_r \quad \text{with n-1 d.f}$$

where n = no. of levels. That is one compares the mean of responses having L at high level (Y_H) with the overall mean of the remaining responses (Y_r).

(ii) Interaction Effects

To determine whether there was any interaction between any two variables, e.g., L and M and the effect on EC, the following treatment was used:

$$1/4[(L_L M_L C_L + L_L M_L C_H + L_H M_H C_L + L_H M_H C_H] - [L_L M_H C_L + L_L M_H C_H + L_H M_L C_L + L_H M_L C_H)]$$

i.e. one adds together responses having the two variables either at 'high' or 'low' levels and subtracts the sum of the other combinations from this value.

Interpretation of result

A result of zero will indicate no interaction whilst a significant departure from zero implies that the two variables were probably interacting with each other to affect EC value. Likewise for the independent effects, the amount by which the result of the treatment departed from zero is a quantitative measure of the individual effects of the variable on EC of the liposomes.

Material and methods

Materials

Egg phosphatidylcholine (egg PC, EPC type V11-E) 500 mg in chloroform/methanol solution and dipalmitoylphosphatidyl choline 100 mg in chloroform/methanol solution (DPPC, Synthetic) were obtained from Lipid products, South Nutfield, England. Vitamin K_1 (phylloquinone), 1 gm in chloroform solution was from Hoffmann La Roche, A.G. Basel. High purity nitrogen gas was purchased locally. Monobasic sodium phosphate, chloroform, dibasic sodium phosphate were products of Sigma chemicals, St. Louis Mo, USA.

Phase contrast microscope (Leltz- wetzlar, Germany), Beckman UV-VIS spectrophotometer and Rotary evaporator (speed vac sc 110, SAVANT) were variously employed for size characterization, to monitor drug concentration and for drying of lipids, respectively. A thermostated shaking water bath and ultracentrifuge (Beckman's) were also utilized for hydration of lipids and separation of free drug from liposomally entrapped drugs.

Methods

Preparation of Vitamin K_1 liposomes using Egg Lecithin

About 2ml of chloroform stock solution containing 20 mg (26μM) of egg phosphatidylcholine (egg PC) was taken into a glass flask. 4μL chloroform stock solution of vitamin K_1 containing about 2mg (4.4μM) of the drug was added and the resulting system mixed thoroughly. This was then placed on a rotary evaporator to remove the organic solvent from the lipid/ drug mixture, which resulted in the formation of a thin film on the inner wall of the flask. Evaporation was done at room temperature (ca. 30°C). Rehydration of the dried lipids in buffer of pH 7.4 (phosphate buffered saline, PBS) was done using two operations: One batch of liposomes was resuspended in phosphate buffered saline (pH 7.4) with gentle shaking for about 2 hours. A second batch of vesicles was hydrated in PBS (pH 7.4) by vigorous vortexing every 20 min. for the same period (i.e. 2 hours). Each vortex-mixing was for about 6 min. The liposomal preparations were labelled appropriately and protected from light throughout the treatment period by shutting the drapes, wrapping in metal foils and kept in the dark.

The procedure was repeated using chloroform stock solution of vitamin K_1 containing 8.8, 13.3, 17.1 and 22 μM each to 26μM of egg PC in separate experiments. After the treatment periods, the liposomal drug suspension was separated from free drug by centrifugation, characterized and analysed.

Preparation of Vitamin K_1 using dipalmitoyllecithin

Dipalmitoyllecithin, 10 mg (about 13.6 μM) and five concentration levels of vitamin K_1 (4.4, 8.8, 13.3, 17.1 and 22 μM) were dissolved in chloroform. The chloroform was removed from the lipid mixture by rotary evaporation under nitrogen at about 37°C. The lipid/drug co-evaporation produced a thin film on the inner wall of the flask. The dried film was re-dispersed in buffer, pH 7.4 at 60°C by either mixing in a shaking water bath maintained at 60°C or by vortex mixing.

Separation of drug-loaded liposome

The liposomal suspensions were washed three times in the same buffer (2 ml of PBS, pH 7.4) by centrifugation at 10,000g for 5 min. in a refrigerated centrifuge (4°C). Supernatant containing drug was removed after each centrifugation step and liposomal pellet re-suspended in 2 ml of fresh buffer. After the third washing, and just before use, the liposomal pellets were resuspended in about 2ml of fresh buffer. All preparations were, thereafter, stored at 4°C until used.

Microscopic study of liposomes

The liposomal suspensions were subjected to size analysis under a light microscope fitted with a calibrated ocular micrometer. The shapes and lamellarity were also studied using a phase-contrast microscope. One hundred liposomes were randomly sized in each experiment.

Drug encapsulation capacity determination

From the centrifuged preparation suspended in phosphate buffered saline (pH 7.4), 1ml sample was withdrawn into a test-tube. An equal amount of iso-osmotic isopropyl alcohol was then added to disrupt the phospholipid vesicles. The volume was made up to 10ml with PBS. After appropriate dilution, each liposomal sample was assayed for drug content

using UV-Vis spectrophotometer at 249nm. The encapsulation capacity (EC) was calculated from the following equation, on the basis of the initial lipid weight.

$$EC = C_a/C_o \cdot C_L \quad \text{Equation 1}$$

where C_a and C_o denote the amounts of liposome associated and initially added molar drug concentration, respectively, and C_L is the molar concentration of lipids in the liposomes.

Results and discussion

Macroscopy and Microscopy

In this study, liposomes formed by the cast film method [12] appeared as milky dispersion which did not froth upon shaking. This could be due possibly to free molecule adsorption at the air/water interphase [13]. The liposome suspensions were heterogenous in size and shape. Most were "onion-skinned" with several concentric bilayers, which suggests the formation of predominantly multilamellar vesicles (MLVs). The average vesicle size was found to be 2.37μm. A positive moderate degree of skewness (0.285) was observed. The mean particle sizes of the various formulations studied are shown in Table 1. In the case of egg PC non-uniform aggregates were observed a confirmation of the fluid nature of egg PC at room temperatures attributable to the low transition temperature of PC [14]. The annealing of formed egg PC liposomes during the hydration process may also be responsible for some of the irregular shapes noted.

Encapsulation capacity

A second order reciprocal equation of the form:

$$EC = A_o + A_1/C_o \quad \text{Equation (2)}$$

was found to adequately represent the relationship between the encapsulation capacity, EC and the combination of formulation variables present, with a coefficient of determination, r^2 value of ≥0.9. A_o is the error term and A_1 is the amount of drug encapsulated per mole of lipid whilst C_o is the initial drug concentration added to the lipid mixture. A_o and A_1 are thus dependent on the combination of variables present. Figure I shows the effects of the variables, L, M and C at their 'low' and 'high' levels on EC.

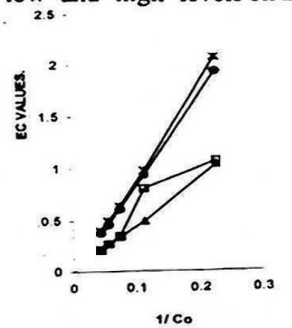

Fig. I. EFFECT OF NATURE OF LIPOSOME MEMBRANE(L), METHOD OF AGITATION(M) AND DRUG CONCENTRATION (Co) ON ENCAPSULATION CAPACITY (EC) OF VESICLES.

Key: $1/C_o L_L M_H$ — $1/C_o L_L M_L$ — $1/C_o L_H M_L$ — $1/C_o L_H M_H$ —

Co = initial drug concentration.
L_L, L_H = nature of liposome membrane at "low" and "high" levels
M_L, M_H = method of agitation at "low" and "high" levels

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To study the effects of the variables on the EC of the liposomes, the value of EC for the liposomal suspensions at $1/C_o$ of 0.045 and 0.227 (i.e 'low' and 'high' values of $1/C_o$) as presented in Table 2 was employed, using the appropriate expressions.

Table 2: 2^3 factorial experimental matrix of the effects of L, M and C on EC of Vitamin K_1 loaded liposomes.

Encapsulation Capacity, EC.				
Variables	L_HM_L	L_LM_L	L_LM_H	L_HM_H
C_L	2.067	1.068	1.034	1.93
C_H	0.399	0.218	0.210	0.378

From the results (Table 3), the ranking of the individual effects of the variables on the encapsulation capacities, EC of liposomes was C>L>M. The positive effect on EC when L

Table 3: Quantitative effects of L, M and C on EC

Variable	Independent Coefficient of EC
L	+0.561
M	-0.050
C	-1.224

($p < 0.001$)
Ranking: C > L >> M
Key:
L = Membrane lipid
M = Method of agitation
C = Initial drug concentration

(lipid) was changed from a 'low' level (EPC) to a 'high' level (DPPC) is probably due to the difference in their 'fluidities' whereby egg-lecithin is composed of highly unsaturated lipids that are 'leaky' when employed at temperatures above their phase transition temperatures [15]. On the other hand, DPPC membranes are considered to be in 'solid' state at conditions of room temperature and the absence of double bonds in the paraffin chains probably prevented leakage of entrapped solute [15]. These observations are in agreement with the reports of Keogh and Davis [16] who observed that the chemical nature of the lipid has a bearing on the transition temperature and thus fluidity of the membrane. The method employed in this study to quantitate the effect of fluidity of membrane on drug entrapment revealed a clear positive effect on entrapment with increase in solid state of membranes at room temperature.

The retention of vitamin K_1 was greater in liposomes prepared using DPPC than those prepared with egg PC. These results are in agreement with the literature [15, 16]. When liposomes are 'fluid', entrapped materials tend to leak out more rapidly than when 'solid' liposomes are used. The small negative effects of M (agitation method employed to re-suspend thin film of lipid-drug complex) on EC when M was changed from 'low' (gentle hand shaking) to 'high' (vigorous vortex mixing) may be due to the fact that liposomes obtained with 'low' M had statistically higher mean diameters with probable higher entrapped volumes compared to liposomes similarly prepared using vigorous vortexing. Maximum encapsulation values of a suspension of lipid vesicles is thought to be a function of liposome size, the number of lamellae [17] and the drug's lipophilicity [18].

Furthermore, the drug (vitamin K_1) in addition to solubilization in the lipid bilayers may also be suspended in the aqueous volume of the vesicles in the particulate form.

However, the large negative effect of C, drug concentration, on EC when it is increased from low to high seems to suggest a saturable process.

From the interaction effects in Table 4 (L-C>>M-C> L-M> L-M-C), it can be seen that the effects of lipid nature, L, and initial drug concentration, C, added to form liposomes is several orders of magnitude higher than M-C, L-M and L-M-C interaction on EC. This implies that the initial drug concentration (or drug/lipid ratio) as well as the nature of lipid comprising the liposome membranes are critical to EC of liposomes in this study. Whilst the types/method of agitation are probably of secondary importance, the significant interaction of C with the other two-way interaction variables also underscores the importance of the need to monitor closely drug/lipid ratio for an optimal drug entrapment in liposomes.

Table 4: Quantitative effects of variables' combination on encapsulation capacity (interaction coefficient)

Variables Combinations	Interaction Coefficients	Ranking
L-M	-0.029	L-C>>M-C
L-C	-0.387	L-M>L-M-C
M-C	0.046	
L-M-C	0.0225	

($p < 0.001$)
Key:
L-M= Lipid – agitation method interaction
L-C= Lipid – initial drug concentration interaction
M-C=Agitation method – initial drug concentration interaction
L-M-C= Lipid – agitation method- initial drug concentration interaction.

Conclusion

The ranking of the effects of the formulation variables of vitamin K_1 liposomes on their entrapment capacity (EC values) provide some indication of the variables' independent and interaction influence. The results indicate that the initial drug concentration and the drug/lipid ratio interaction (L-C) affect the encapsulation capacity of vitamin K_1 in liposomes more than other variables used in this study. The order of interaction effects found are L-C>> M-C> L-M> L-M-C.

From these results, it can be concluded that the quantitation of the effects of variables of a liposomal formulation can be employed in pre-formulation studies in the development of a liposome-based dosage form. Of particular note is that proper formulation of liposomes is important for good encapsulation of drug materials.

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