ELSEVIER

International Journal of Pharmaceutics 143 (1996) 171–177

international journal of pharmaceutics

Factors influencing the encapsulation of thioguanine in DRV liposomes

Elisenda Casals, Montserrat Gallardo, Joan Estelrich*

Unitat de Fisicoquímica, Facultat de Farmàcia, Universitat de Barcelona, Avda. Diagonal s/n, 08028-Barcelona, Catalonia, Spain

Received 12 May 1996; accepted 14 August 1996

Abstract

Thioguanine, a chemotherapeutic drug employed in the treatment of leukemia, is, like other amphiphilic drugs, very permeant and presents a high ability to escape from the liposomal membrane. For this reason, it is very important to know which parameters can enhance the relatively low encapsulation of thioguanine. In this way, the influence of four factors (pH, content of cholesterol, charge of lipids, and time of sonication) on the encapsulation of such drug in dehydration-rehydration liposomes was studied using a 2^4 factorial design. In this study, the maximal encapsulation efficiency obtained was 14.07 mmol/mol of lipid and time of sonication was the unique factor whose influence on the encapsulation was statistically significant ($P < 0.05$). The energy input concomitant to higher periods of sonication seems to facilitate the entrapment of the drug into the bilayer. Among the two-way interactions, time·charge and pH·charge presented levels of significance less than 0.05. In liposomes with negative charge (10% phosphatidic acid), time of sonication barely influenced on the encapsulation, but in stearylamine-containing liposomes, higher times of sonication were necessary to achieve a better yield of encapsulation. More complex was the effect of the pH–charge interaction. At pH 4.7, liposomes with phosphatidic acid favoured the encapsulation, while at pH 7.4, liposomes with stearylamine encapsulated more drug. The different extent of encapsulation in the function of pH and charge only could be explained by the different charge born by the positive and negative liposomes depending on the pH.

Keywords: Liposomes; Thioguanine; Encapsulation; Dehydration-rehydration vesicles; Factorial design

1. Introduction

Thioguanine, a chemical analog of the physiological purines, guanine and hypoxanthine, is a chemotherapeutic drug. It is converted intracellularly to ribonucleotides which have multiple metabolic effects, resulting in a sequential blockade of the synthesis and utilization of purine nucleotides, since thioguanine ribonucleotides are incorporated into the DNA and RNA of bone marrow cells. Thioguanine is used primarily as a

* Corresponding author.

PII S0378-5173(96)04701-1

component of various chemotherapeutic regimens for remission induction in acute and chronic myelogeneous leukemia. It is not effective in the treatment of Hodgkin's disease and related lymphomas, multiple myeloma, or most solid tumors. The major adverse effect of thioguanine is hematologic toxicity, which is dose related and manifested by leukopenia, thrombocytopenia, and anemia. Jaundice has also occurred in some leukemic patients receiving thioguanine.

Liposomes have been investigated since 1970 as a system for the delivery or targeting of drugs to the specific sites in the body. In cancer chemotherapy, accumulated in vivo evidence has indicated clearly that some liposome-entrapped drugs exhibit superior pharmacological properties to those observed with conventional formulations.

In a previous paper (Foradada and Estelrich, 1995) the encapsulation of thioguanine in neutral liposomes was described. From this, it was inferred that pH 4.7 afforded more efficient encapsulation than the other pH values checked (7.4 and 9.2). Such pH corresponded to the maximal apparent partition coefficient and, hence, to the maximal neutrality of the molecule. On the other hand, dehydration-rehydration of vesicles (DRV) was found to be the optimal method for encapsulation of thioguanine irrespective of the pH. Based upon the foregoing facts, now we have planned the study of the influence of several factors (namely charge of lipids, cholesterol content, pH and sonication time) on the encapsulation efficiency of thioguanine in DRV liposomes. In order to check the simultaneous influence of all the factors as well as the interactions among them a statistical experimental design was used. A factorial design gives much more information and reaches the optimum conditions in fewer experiments than does classical experimentation which scrutinizes the variables one at a time (Wold, 1991). Factorial designs are widely used in experiments involving several factors on a response. The 2^k design is particularly useful in the early stages of experimental work, when there are likely to be many factors to be investigated. It provides the smallest number of runs with which k factors can be studied in a complete factorial design (Montgomery, 1991).

2. Materials and methods

2.1. Materials

Thioguanine was purchased from Fluka (Germany). In the preparation of liposomes Lipoid S-100 (Lipoid KG, Ludwigshafen, Germany), a mixture of lipids, whose main component is soy bean phosphatidylcholine (PC), was used. Other lipids such as stearylamine (STE), phospathidic acid (PA) or cholesterol (CHOL) were purchased from Sigma (USA). Buffer solutions (acetate pH 4.7, citrate pH 6.2, phosphate pH 7.2, and Hepes pH 7.4) were made at 10 mM concentration, containing 145 mM NaCl. In buffer solutions employed to solubilize the drug, the salt concentration was corrected in order to maintain the isotonicity (310 ± 10 mOsm/kg). Water was double-distilled, the last time in the presence of potassium permanganate, in borosilicate apparatus, and then purified through a Milli-Q system (Millipore, USA). Hydrogen peroxide and organic solvents (dimethylsulfoxid (DMSO), methanol and chloroform) were obtained from Merck (Germany) and used without purification. C18 Sep-Pak cartridges (Waters-Millipore, USA) were used for the liquid-solid extraction.

2.2. Methods

2.2.1. Solubilization of the drug

A weighted amount of thioguanine was dissolved in DMSO at 50 mM. Finally, the solution was diluted with a suitable buffer solution (acetate, citrate or Hepes) until obtaining the final concentration (0.5 mM).

2.2.2. Liposome preparation

The dehydration-rehydration liposomes (DRV) were prepared according to the method of Kirby and Gregoriadis (1984). In brief, a suspension of multilamellar liposomes at 10 μmol/ml lipid concentration was sonicated three times at different son ication times (30, 60 and 120 s) in an ultrasonicprove (Labsonic U, USA) to obtain small unilamellar liposomes (SUV). This step was performed in an ice bath and under nitrogen atmosphere. The resultant SUV suspension was

centrifuged at 2000 rpm for 15 min to remove titanium debris and large lipid aggregates. To 2 ml of SUV was added 1 ml of the thioguanine solution, frozen and lyophilized on a Flexy-Dry (USA) freeze-drier for 16–20 h. The lyophilized preparation was rehydrated with 1 ml of bidistilled water.

2.2.3. Liposome purification

DRV liposomes containing encapsulated drug were separated from free drug by centrifugation in a 50 Ti type rotor of a Beckmann L8-60 M ultracentrifuge. A 5 ml suspension volume was centrifuged for 30 min at 20 000 rpm ($= 35\,000 \times g$) at 7°C. This washing step was repeated twice.

2.2.4. Size analysis

Determination of vesicle size distribution has been made by dynamic light scattering employing an Autosizer IIc photon correlation spectrometer (Malvern Instruments, United Kingdom). The sample, contained in a 5 ml plastic cuvette, was placed in a thermically packed sample holder at 25°C. The light source, a helium-neon laser of wavelength 632.8 nm and 5-mW, was focused onto the sample and scattered light was detected at 90° to the incident beam by a photomultiplier tube, which is connected to a quantum photometer. Fluctuations in scattered light intensity generated by the diffusion of vesicles in solution were analyzed and the autocorrelation function obtained via a Malvern 7032-N, 72-channel multibit correlator. As DRV liposomes do not all have the same size but rather follow a size distribution function $N(R)\mathrm{d}R$ (meaning that a fraction $N(R)\mathrm{d}R$ of the particles present in the suspension have a radius between R and $R + \mathrm{d}R$) it is necessary the use of a mathematical method to solve the problem of the polydispersity (Ostrowsky, 1993). In this case, we have employed the exponential sampling method, which does not assume any particular form of distribution (Ostrowski et al., 1981).

2.2.5. Lipid assay

Lipid was assayed spectrophotometrically by the Steward-Marshall (1980) method. It was confirmed in advance that thioguanine had no influence on the color development. For each lipid composition the corresponding calibration curve was carried out.

2.2.6. Drug assay

Disruption of the membrane structure was carried out by liquid solid extraction, employing C18 Sep-Pak cartridges (Waters-Millipore, USA). After breaking down the vesicles, the aqueous phase containing the drug was oxidized, the fluorescence being determined at 330/415 nm as described elsewhere (Foradada et al., 1994). The assay was performed using an Hitachi F2000 fluorescence spectrometer (Hitachi, Japan) and calibrated using known quantities of thioguanine.

From the amount of drug and lipid contained in the vesicles, the encapsulation efficiency was determined as the relationship of mmol of drug to mol of lipid present. This parameter was used to compare the ability of different compositions to encapsulate thioguanine.

2.2.7. Factorial design

A complete 2^4 factorial design at two levels with three center points was used. As a potential concern in the use of two-level factorial designs is the assumption of linearity in the factor effects, replicating certain points at the center will provide protection against curvature as well as allow an independent estimate of error to be obtained. Table 1 shows the experimental conditions chosen.

Table 1
Factors and levels investigated in the optimization of the encapsulation of thioguanine in liposomes

Variables (factors)	Levels		
	Low (−)	Medium (0)	High (+)
x_1 = pH	4.7	6.1	7.4
x_2 = cholesterol (% molar ratio)	0	33	50
x_3 = charge of lipid	negative	uncharged	positive
x_4 = time of sonication(s)	30	60	120

Table 2
Matrix and results for the 2^4 factorial design used

Run (order)	x_1	x_2	x_3	x_4	y
1(5)	−1	−1	−1	−1	9.84 ± 1.62
2(14)	1	−1	−1	−1	8.63 ± 0.53
3(16)	−1	1	−1	−1	3.12 ± 0.10
4(4)	1	1	−1	−1	6.13 ± 1.02
5(13)	−1	−1	1	−1	7.03 ± 0.95
6(3)	1	−1	1	−1	6.11 ± 0.71
7(1)	−1	1	1	−1	3.95 ± 0.37
8(12)	1	1	1	−1	9.86 ± 0.71
9(10)	−1	−1	−1	1	7.25 ± 0.35
10(8)	1	−1	−1	1	6.35 ± 0.79
11(2)	−1	1	−1	1	10.88 ± 1.31
12(15)	1	1	−1	1	5.74 ± 0.24
13(6)	−1	−1	1	1	12.85 ± 1.20
14(11)	1	−1	1	1	12.40 ± 0.90
15(17)	−1	1	1	1	8.10 ± 0.24
16(7)	1	1	1	1	14.07 ± 1.31
17(9)	0	0	0	0	7.01 ± 0.63
18(19)	0	0	0	0	7.24 ± 0.55
19(18)	0	0	0	0	6.92 ± 0.14

x_1 = pH; x_2 = cholesterol content; x_3 = charge of lipid; x_4 = time of sonication; y = encapsulation efficiency expressed as mmol of drug/mol of lipid. The order of randomization is shown in brackets.

2.2.8. *Analysis of data*

Each data point shown in Section 3 is the mean of three determinations in duplicate. The values of encapsulation efficiency are given as mean ± S.D. Modeling was obtained by multivariate regression analysis, and the influence of each variable was evaluated by multifactor ANOVA. In both cases the statistic package Statgraphics 7.0 (STSC, USA) has been used.

3. Results

Nineteen experiments have been carried out according to the factorial design 2^4 with three central points of Table 2. Values of encapsulation efficiency are the average of two replicates. As the statistical validity of much of what is deduced, depends on randomization and on the assumption that all the runs are carried out identically, before performing the experiments, it is important to determine a randomized sequence of runs.

From encapsulation efficiency values, a linear model including two-way interactions (1 + 4 + 6 terms) was proposed and multiple linear regression was used to fit the model to the data. Interactions of the 2nd and 3rd order have not been studied due to the complexity of their interpretation. Table 3 shows the regression coefficients obtained with such model and the statistical significance of the factors and their interactions have been determined using analysis of variance on the dependent variable encapsulation efficiency (Table 4).

From Table 3 it can be established that only the time of sonication has a significative effect ($P < 0.05$) on the encapsulation of thioguanine in DRV liposomes. The two-way interactions which were found to be significant at a similar level were: pH–charge and charge–time interactions. In order to adjust the model, non-significant coefficients of two-way interactions were omitted and recalculations were done. Hence, another analysis was carried out after pooling four factors into the error term. This increased the degrees of freedom for the error term from 8 to 12 and consequently enhanced the sensitivity of the statistical analysis of the data. The new model presented the same significant factors but now the determination coefficient (R^2) reached 0.8113, and the adjusted reached 0.7170. Furthermore, the significance level of the linear model (P) decreased to 0.0009.

4. Discussion

The use of liposomes for the delivery of drugs is an attractive possibility since they consist of biodegradable lipid components in a spontaneously forming bilayer configuration, the composition of which can be varied to a marked extent. The use of liposomes as carriers of anti-cancer drugs has been intensively studied.

In this paper, we describe the encapsulation of thioguanine in DRV liposomes under different conditions of pH, charge of lipids, content in cholesterol, and time of sonication. For the simultaneous study of all the factors a two-level factorial design has been used. Two-level designs are appropiate in preliminary experiments to screen a large number of potentially effective factors.

Table 3
Coefficients of regression and levels of significance '*P*' associated to each variable

Independent variable	Coefficient	S.E.	*t*-value	*P*
Constant	8.177895	0.37242	21.9586	0.0000
pH	−0.204375	0.40584	−0.5036	0.6281
Cholesterol	−0.268125	0.40584	−0.6607	0.5274
Charge	0.456875	0.40584	1.1258	0.2929
Time	1.540625	0.40584	3.7961	0.0053
pH–cholesterol	0.381875	0.40584	0.9410	0.3743
pH–charge	1.316875	0.40584	3.2448	0.0118
pH–time	0.113125	0.40584	0.2787	0.7875
Cholesterol–charge	−0.234375	0.40584	−0.5775	0.5795
Cholesterol–time	0.036875	0.04584	0.0909	0.9298
Time–charge	1.469375	0.04584	3.6206	0.0068

R^2 (adjusted) = 0.6350
S.E. = 1.623359; M.A.E. = 0.906953

S.E., Standard error; M.A.E., mean absolute error.

Dehydration-rehydration vesicles have many advantages such as relatively high entrappment efficiency and little potential damage to the entrapped material by organic solvents during preparation. The only disadvantage is the broad size distribution of the vesicles obtained, since this characteristic plays a critical role in the pharmacokinetics of both the liposomes and the entrapped drug in vivo (Hwang, 1987). Size of the vesicles used in this study ranged from 750 to 1800 nm, expressed as *n*-diameter, while their polydispersity ranged from 0.35 to 0.81.

Encapsulation efficiencies obtained are ranging from 3.12 to 14.07 mmol/mol lipid. Highest encapsulation has been achieved with positive liposomes at pH 7.4, containing cholesterol (50%) and sonicated at periods of 120 s. Contrarily, the lowest value has corresponded to negative liposomes, made at pH 4.7, containing also a 50% of cholesterol, and sonicated at periods of 30 s. In a previous paper (Foradada and Estelrich, 1995) the maximal value obtained for the same initial concentration of thioguanine (0.5 mM) was 9.14 mmol/mol. Results shown in Table 2 demonstrate the relatively poor encapsulation of thioguanine in liposomes. The amphiphilic characteristics of this drug accounts for its ability to permeate the lipid membrane.

From an analysis of variance it was established that the unique main effect with statistical significance was the time of sonication. In this way, the average value of encapsulation of samples sonicated at periods of 30 s was 6.63 mmol/mol in front of 9.72 mmol/mol achieved when the vesicles were sonicated at periods of 120 s. The influence of time of sonication on encapsulation can be explained by the energetic input that the sonication represents. A longer sonication time involves the formation of a more homogeneous and smaller population of vesicles, and the drug will distribute better in this kind of liposome. Beyond that, the energy afforded in the sonication process will permit to overcome the possible repulsive interactions between lipid molecules and the drug. As far as the two-way interactions is concerned, Fig. 1 shows the interaction time·charge. We can see that at the low level of lipid charge, or better to say in liposomes with phosphatidic acid, barely there are important differences in the encapsulation. Nevertheless, in stearylamine-containing liposomes (high level of charge) the difference is great and it is necessary to spend large times of sonication in order to achieve higher encapsulation efficiencies. Fig. 2 represents the pH–charge interaction. It shows that although the variable pH does not exert a significant influence when it is considered alone, it does show an influence if it is

Table 4
Analysis of variance of linear model assumed

Source of variation	Sum of squares	DF	Mean square	*F*-Ratio	*P*-value
Model	108.865	10	10.8865	4.13104	0.0280
Error	21.0824	8	2.63529		
Total	129.947	18			
$R^2 = 0.837762$		S.E. = 1.62336			
R^2 (adjusted for DF) = 0.634965		Durbin-Watson test = 2.43878			

DF, Degrees of freedom; S.E., standard error.

considered together with the charge. In this way, the higher encapsulation is obtained at pH 4.7 when phosphatidic acid is forming the membrane. At pH 7.4 the encapsulation is greater in liposomes formed by stearylamine. One could come up with a different extent of electrostatic interactions for explaining the differences in encapsulation. Given that phosphatidic acid presents two ionizable groups with pK_a of 3.9 and 8.3, respectively (Quinn, 1976), it must have a net charge of -0.86 and -1.36 at pH 4.7 and 7.4, respectively. Stearylamine, with a pK_a of 10.6 (Klang et al., 1994), is charged positively in the range of studied pHs. On the other hand, spectrophotometrically measurements afforded a pK_a of thioguanine equal to 8.26 (Foradada and Estelrich, 1995). Based upon this pK_a, we can deduce that thioguanine is present in molecular form (< 0.05% of species with net charge) at pH 4.7, while at pH 7.4 the percentage of ionized drug only reaches the 6%. Thus, we must consider the drug as a neutral species, albeit it can present a zwitterionic structure and, hence, it can undergo some electrostatic interactions with the charged lipids as well as with the zwitterionic PC. However, this does not explain the differences found between the encapsulation by positive liposomes in front of negative liposomes as a function of the pH. Only the different superficial charge that presents phosphatidic acid in function of pH could suggest differences in the process of entrapment.

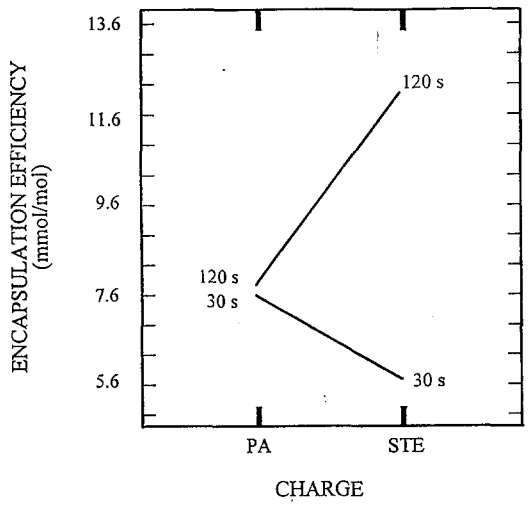

Fig. 1. Effect of the charge–time interaction on the encapsulation efficiency of thioguanine in DRV liposomes.

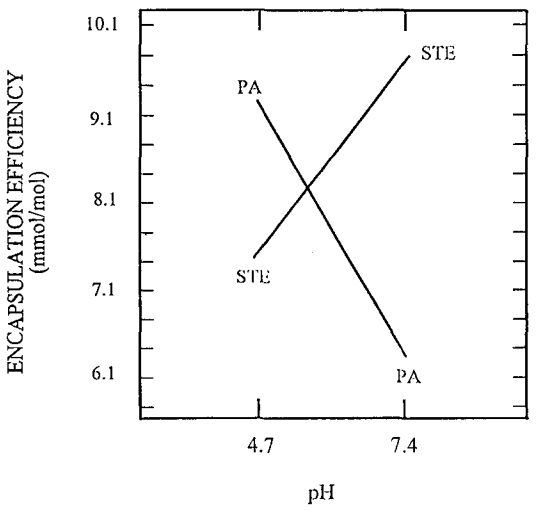

Fig. 2. Effect of the pH–charge interaction on the encapsulation efficiency of thioguanine in DRV liposomes.

In this way, at pH 4.7 liposomes with lesser superficial charge are those formed by phosphatidic acid (− 0.86 units of charge), whereas at pH 7.4 liposomes with stearylamine bear + 1 unit of charge in comparison with the − 1.36 units of charge of negative liposomes.

References

Foradada, M. and Estelrich, J., Encapsulation of thioguanine in liposomes. *Int. J. Pharm.*, 124 (1995) 261–269.

Foradada, M., Gallardo, M. and Estelrich, J., Design and applications of a new fluorimetric assay of thioguanine in liposomes. *J. Pharm. Biomed.*, 12 (1994) 1495–1499.

Hwang, K.J., Liposome pharmacokinetics. In Ostro, M.J. (Ed.), *Liposomes—From Biophysics to Therapeutics*, Dekker, New York, 1987, pp. 109–156.

Kirby, C. and Gregoriadis, G., Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. *Biotechnology*, 2 (1984) 979–984.

Klang, S.H., Frucht-Pery, J., Hoffman, A. and Benita, S., Physicochemical characterization and acute toxicity evaluation of a positively-charged submicron emulsion vehicle. *J. Pharm. Pharmacol.*, 46 (1994) 986–993.

Montgomery, D.C., The 2k factorial design. In *Design and Analysis of Experiments*, 3rd edn., Wiley, Singapore, 1991, Chp. 9.

Ostrowsky, N., Liposome size measurements by photon correlation spectroscopy. *Chem. Phys. Lipids*, 64 (1993) 45–56.

Ostrowski, N., Sornette, D., Parker, P. and Pike, E.R., Exponential sampling method for light scattering polydispersity analysis. *Optica Acta*, 28 (1981) 2988–2994.

Quinn, P.J., *The Molecular Biology of Cell Membranes*, Academic Press, London, 1976, pp. 69.

Steward-Marshall, J.C., Colorimetric determination of phospholipids with ammonium ferrothyocianate. *Anal. Biochem.*, 104 (1980) 10–14.

Wold, S., Chemometrics, why, what and where to next. *J. Pharm. Biomed.*, 9 (1991) 589–596.