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Encapsulation of thioguanine in liposomes

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Abstract

Thioguanine, a chemotherapeutic drug employed in the treatment of leukemia, was encapsulated in three kinds of liposomes: extrusion, ethanol injection and dehydration-rehydration vesicles. The degree of entrapment was examined at three different concentrations, 1, 0.1 and 0.01 mM, and three pH values, 4.7, 7.4 and 9.2. Thioguanine had a considerable ability to cross the lipid bilayer, especially if the molecule presented a net charge. Like the amphiphilic drugs it was very permeant. Concerning the kind of liposomes, dehydration-rehydration of vesicles was found to be the optimal method for encapsulation of thioguanine irrespective of the pH. At pH 4.7 an encapsulation efficiency of 12 mmol/mol of lipid was elicited. The other methods never entrapped more than 3 mmol/mol of lipid. This behaviour can be explained by the formation in the drug of intermolecular bonds by hydrogen bridges during the process of lyophilization. The product formed might encounter greater difficulty in escaping from the vesicle than the non-bonded thioguanine. On the other hand, pH 4.7 also afforded more efficient encapsulation than the other pH values. Furthermore, this pH corresponds to the maximal apparent partition coefficient, and, consequently, to the maximal neutrality of the molecule. At this point, thioguanine is present as a neutral or as zwitterionic species. From these findings it can be inferred that entrapped thioguanine, as a zwitterionic form, is associated with the membrane lipids by means of electrostatic interactions with the zwitterionic phospholipid.

Keywords: Liposome; Thioguanine; Encapsulation; Extrusion; Ethanolic injection; Dehydration-rehydration vesicle

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 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

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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. There is substantial evidence that, in experimental animals at least, drug concentrations in certain tumours are higher than in neighbouring normal tissues. This can be account for one or more of the following factors: (a) higher endocytic activity of some tumour cells; (b) diffusion of drugs from liposomes either during circulation or after they have been lodged in tissues near tumours; (c) engulfment of liposomes by circulating monocytes and migration of the latter to tumours (Gregoriadis and Florence, 1993). However, the use of liposomes in cancer chemotherapy appears to be more necessary when the aim is to reduce the intrinsic toxicity while maintaining a cytotoxic effect. In this way, some cytostatic drugs have been encapsulated in liposomes, for example, doxorubicin, daunorubicin, *cis*-platinum, mitoxantrone, methotrexate, vinblastine, vincristine, actinomycin D, bleomycin and 5-fluorouracil. Among them, experiments with anthracycline cytostatic drugs (doxorubicin and daunorubicin, mainly) have clearly shown reduced cardiotoxicity and dermal toxicity, and better survival of experimental animals compared with controls receiving the free drug (Gregoriadis and Florence, 1991). The success of such experiments has allowed liposome-entrapped anthracyclines to be used in clinical trials. For instance, in phase II studies, doxorubicin-containing liposomes exhibited substantial activity in metastatic breast cancer with no apparent cardiotoxicity (Rahman et al., 1993).

As far as thioguanine is concerned, there is no information about its encapsulation in liposomes. The entrapment of a related drug, mercaptopurine, has been described elsewhere (Tsujii et al., 1976; Kano and Fendler, 1977), although the values obtained were not very large. Based upon the above, the aim of this work was the entrapment of thioguanine in liposomes obtained by different methods under several pH and concentration conditions. In this way, we have studied entrapment in liposomes achieved by extrusion (Hope et al., 1985), ethanol injection (Batzri and Korn, 1973) and the dehydration-rehydration of vesicles (Kirby and Gregoriadis, 1984) at pH values of 4.7, 7.4 and 9.1 at three drug concentrations, namely, 1, 0.1 and 0.01 mM. Furthermore, the partition coefficient of the drug has been exploited in order to relate the physicochemical properties to the encapsulation parameters.

2. Materials and methods

2.1. Materials

Thioguanine (lot 269521) 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 phosphatidylcholine, was used. Buffer solutions (acetate pH 4.7, Tris-HCl pH 7.4 and borate pH 9.1) 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 final 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 (isopropanol, methanol, butanol and chloroform) were obtained from Merck (Germany) and used without further purification. Octanol (> 99%) was achieved from Carlo Erba (Italy). C18 Sep-Pak cartridges (Waters-Millipore, USA) were used for liquid-solid extraction.

2.2. Methods

2.2.1. Solubilization of the drug

A weighed amount of thioguanine was dissolved in 0.05 N NaOH. After complete solubilization, 50% acetic acid was added until the desired pH value was achieved. Finally, the solution was diluted with suitable buffer solution (acetate, borate or Tris-HCl) to give the desired final concentration (1, 0.1 or 0.01 mM).

2.2.2. Determination of partition coefficient

Partition coefficients were determined by the flask-shaking method (Purcell et al., 1973). As

Table 1
Absorptive characteristics of thioguanine for different pH values and solvents

Solvent	Molar absorptivity ϵ (M^{-1} cm^{-1})	λ_{max} (nm)
Aqueous solution at pH 2	17100	348
Acetate buffer pH 4.7	18500	342
Tris-HCl buffer pH 7.4	18300	342
Borate buffer pH 9.3	14800	324–326
Aqueous solution at pH 12	15900	322
Methanol-acetate 2:1 (v/v)	20500	346
n-Butanol	19400	348

organic phases, *n*-butanol and *n*-octanol both saturated with the corresponding aqueous phase were used; as aqueous phases, the same buffer solutions saturated with organic phases were used for all subsequent work.

Thioguanine was dissolved directly in the organic-saturated buffer at 100 μM concentration. The final concentration was checked, and corrected if necessary, determining its absorbance (A) at the wavelength of maximal absorption (see Table 1). A volume of this solution was thoroughly mixed with the same volume of buffer-saturated organic phase, the mixture was stirred for 120 min at room temperature and then set aside for 30 min to facilitate phase equilibrium being attained; then, the phases were separated by centrifugation. An aliquot was accurately removed from the aqueous phase and its absorbance measured (A'). The apparent partition coefficient (P') was obtained from $P' = (A - A')/A'$.

2.2.3. pK_a determination

The pK_a of thioguanine was determined by spectrophotometry according to the equation pK_a = pH − log[molecular]/[ionized]. The mole ratio of molecular species (neutral and/or zwitterionic molecule) to ionized species can be determined from absorbance measurements in a series of buffered solutions of known pH at the analytical wavelength of 348 nm, where the greatest difference between their absorbances is seen.

2.2.4. Liposome preparation

Extruded liposomes (EXT) were obtained in the sequential mode. Briefly, 40 μmol of Lipoid were dissolved in chloroform and the solution dried in a rotovapor under a reduced pressure. Lipid was dispersed in 4 ml of drug solution by vortex agitation and bath sonication. The multilamellar vesicles produced above were downsized to form oligolamellar vesicles by extrusion at 37°C in an Extruder device (Lipex Biomembranes, Canada) through polycarbonate membrane filters of variable pore size under nitrogen pressures of up 55×10^5 Nm^{-2}. Liposomes were extruded in a three-step extrusion: first, three consecutive extrusions through a 0.8 μm pore diameter polycarbonate filter (Nucleopore, USA), followed by three consecutive extrusions through a 0.4 μm pore diameter filter and, finally, the resultant liposome suspension was extruded four consecutive times through a 0.2 μm filter.

Liposomes obtained by ethanol injection (INJ) were prepared using a 40 mg/ml lipid concentration. This lipid concentration affords a vesicle population with an average diameter in number of 50 nm and a polydispersity ranging from 0.30 to 0.36 as determined elsewhere (Pons et al., 1993). Thioguanine was dissolved in warm ethanol together with the lipid and 0.75 ml of such a solution were rapidly injected into 10 ml of a magnetically stirred buffer solution. Therefore, 1 mM drug concentration in the ethanol solution is equivalent to 75 μM concentration in the bulk.

Dehydration-rehydration liposomes (DRV) were prepared from small unilamellar vesicles (SUV) obtained by sonication of multilamellar vesicles at 10 μmol/ml lipid concentration. SUV were mixed with an equal volume of the thioguanine solution, frozen and lyophilized on a Flexy-Dry (USA) freeze-drier for 16–20 h. Controlled rehydration was carried out at room temperature by addition of distilled water followed by the corresponding buffer, with vigorous vortexing and incubation at room temperature for 30 min between additions.

2.2.5. Liposome purification

DRV liposomes containing encapsulated drug were separated from free drug by centrifugation

in a 50 Ti type rotor of a Beckman L8-60 M ultracentrifuge. A 5 ml suspension volume was centrifuged for 30 min at 20 000 rpm (= 35 000 × g) at 7°C. This washing step was repeated twice.

Extrusion and ethanol injection liposomes were separated from free drug by the mini-column centrifugation technique (Fry et al., 1978). Excess fluid was first removed from the Sephadex G-50 beads by centrifugation, and the unpurified liposomes were applied to the column bed. Centrifugation was repeated to force the liposomal material through the column into a test tube, while free drug was retained quantitatively in the Sephadex.

2.2.6. Size analysis

Determination of vesicle size distribution has been made by dynamic light scattering employing an Autosizer IIc photon correlation spectrometer (Malvern Instruments, UK). 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 was 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.

For sizing vesicles obtained by extrusion or ethanol injection, which afforded a unimodal vesicle distribution, the method of cumulant analysis (Koppel, 1972) was applied, while for sizing DRV vesicles (a polydisperse system) model-independent analysis, which does not assume any particular form of distribution, was used.

2.2.7. Lipid assay

Lipid was assayed spectrophotometrically according to the method of Steward-Marshall (1980). It was confirmed in advance that thioguanine had no influence on the color development.

2.2.8. 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 a 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 μmol of drug to mol of lipid present. This parameter was used to compare the ability of different liposomes to encapsulate thioguanine.

2.2.9. 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 $\pm$ S.D. Molar absorptivity and pK_a values were obtained by regression analysis employing the Microstat statistic package (Ecosoft, USA). Method, pH and concentration data were evaluated by multifactor ANOVA employing the Statgraphics statistic package (STSC, USA).

3. Results

3.1. Physicochemical properties

Drug solubility study has demonstrated that thioguanine is rather soluble in organic solvents such as isopropanol, butanol, hot methanol or ethanol (50°C) at concentrations above 100 μM. This relatively high affinity for organic media is a consequence of the amphoteric character of thioguanine.

As to the absorptive properties of the drug, the absorption of electromagnetic radiation is mainly due to $\pi \rightarrow \pi^*$ transitions. Thioguanine suffers a slightly hypsochromic shift as the polarity of the solvent increases. This behaviour is attributed to the greater localization of electrons in the ground state in comparison with the electrons in the excited state when the dielectric constant of solvent increases. A hyperchromic effect was also observed in non-polar solvents.

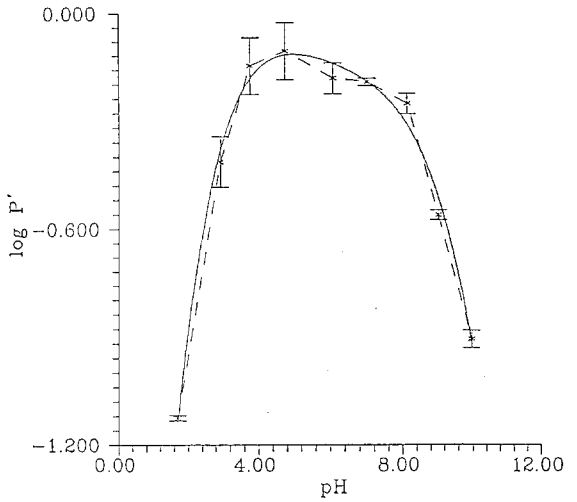

Fig. 1. Apparent partition coefficient of thioguanine at several pH values. Bars represent the standard error of the average value.

On the other hand, the absorption of the molecule is pH-dependent, showing a hypsochromic shift from 348 to 322 nm with increasing pH as can be seen in Table 1. The isosbestic point can be found at 329 ± 1 nm.

Partition coefficients were determined over the pH range between 2 and 10 with an accuracy of ±0.1. Water solutions were prepared from a two-solution buffer system (solution A: 0.2 M anhydrous boric acid and 0.05 M citric acid monohydrate; solution B: 0.1 M trisodium phosphate dodecahydrate) as indicated elsewhere (Silberman, 1992). Buffer-saturated *n*-octanol was the organic phase. Fig. 1 was obtained by plotting the log P' values in front of pH of buffer solution. The amount of drug transferred from the aqueous to the organic phase was maximal at pH 4.74 (log $P' = -0.106 \pm 0.081$). Below and above this pH, the partition coefficients decrease, indicating that the polarity of the drug is greater, and therefore, minor transference.

Since the apparent equilibrium partition coefficients in liposomes are greater than log P' values (Rogers and Choi, 1993), we have also used *n*-butanol as the oil phase. The physicochemical properties of *n*-butanol make it a medium closer to lipid than *n*-octanol. For instance, pure *n*-butanol has a dielectric constant of 17.16 and this

Table 2
Partition coefficients of thioguanine in buffer/*n*-butanol

Aqueous phase	Partition coefficient ($P \pm$S.D.)	Log P' (± S.D.)
Acetate buffer	3.643 ± 0.304	0.561 ± 0.035
Tris-HCl buffer	1.902 ± 0.080	0.279 ± 0.018
Borate buffer	0.289 ± 0.050	−0.539 ± 0.029

value reaches 21.29 if it is water saturated (dielectric constant: 78.54 for water and 9.78 for *n*-octanol). As can be seen in Table 2, log P' values in *n*-butanol are much greater than in *n*-octanol, log P' being extremely high in acetate buffer.

Fig. 2 shows the plot of absorbance as a function of pH of the solution at 348 nm. Employing for the neutral thioguanine molar absorptivity values of 17 100 and 7300 M^{-1} cm^{-1} for 348 and 322 nm, respectively, and for the ionized molecule values of 2100 and 15 900 M^{-1} cm^{-1} for 348 and 322 nm, respectively, a plot of the pH of the solutions as a function of log[molecular]/[ionized] yielded a straight line with an intercept on the pH axis (at log[molecular]/[ionized] = 0) equal to pK_a (inset, Fig. 2). The pK_a obtained was 8.26 ± 0.03.

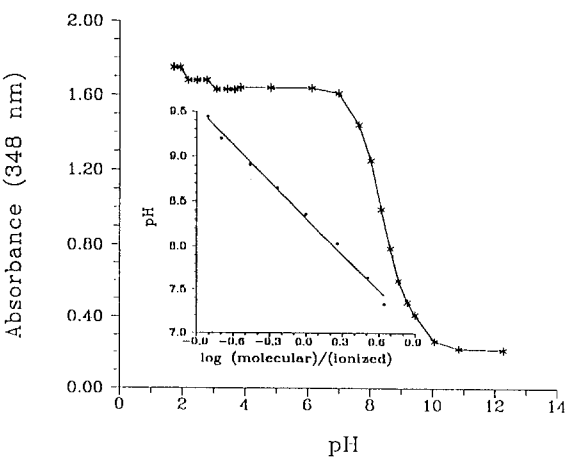

Fig. 2. Plot of absorbance at 348 nm as a function of pH. (Inset) Plot of pH as a function of the ratio between molecular and charged species.

3.2. Encapsulation efficiencies

Table 3 lists the encapsulation efficiencies obtained with the different types of vesicles as well as their size characteristics, namely, average diameter in number (n diameter) and polydispersity. INJ liposomes present an n-diameter and polydispersity values greater than those obtained with empty liposomes (broadly speaking, 50 nm and 0.3 for size and polydispersity, respectively), although according to such size it is logical to suppose that these vesicles are uni-or oligolamellar. The diameters of the other vesicles ranged within the values expected. The extrusion technique yielded monodisperse populations of unilamellar vesicles, irrespective of their drug concentration and pH value showing n-diameters less than 200 nm. Moreover, it is known that EXT liposomes obtained from phosphatidylcholine and extruded through 200 nm membranes present two or three lamellae and, hence, belong to the unilamellar type. DRV liposomes are extremely large (under the above conditions any n-diameter was greater than 1 μm) and usually heterogeneous.

With respect to the encapsulation efficiency, we can observe in Table 3 that DRV liposomes afforded the highest values in comparison with other liposomes. INJ and EXT liposomes never exceeded an encapsulation efficiency of 3 mmol of thioguanine per mol of lipid, whereas DRV liposomes were able to encapsulate such rela-

Table 3
Size parameters and encapsulation efficiency of thioguanine in the different types of liposomes studied

Method	pH	Initial drug concentration (μM)	Encapsulation efficiency (mmol/mol) (mean ± SD)	Vesicle size (nm) (mean ± SD)	Polydispersity
EXT	4.7	1	2.63 ± 0.48	146 ± 9	0.13
		0.5	2.26 ± 0.24	139 ± 10	0.12
		0.1	1.87 ± 0.19	138 ± 18	0.13
	7.4	1	1.24 ± 0.19	155 ± 5	0.13
		0.5	1.10 ± 0.28	147 ± 5	0.09
		0.1	0.77 ± 0.14	158 ± 10	0.11
	9.2	1	1.69 ± 0.60	150 ± 20	0.13
		0.1	0.84 ± 0.19	153 ± 4	0.09
INJ	4.7	1	2.84 ± 0.65	59 ± 10	0.44
		0.5	2.88 ± 0.16	49 ± 7	0.63
		0.1	2.76 ± 0.19	48 ± 8	0.55
	7.4	1	1.00 ± 0.10	62 ± 13	0.50
		0.5	0.76 ± 0.12	64 ± 8	0.53
		0.1	0.42 ± 0.08	64 ± 6	0.50
	9.2	1	0.91 ± 0.10	66 ± 20	0.45
		0.5	0.78 ± 0.10	53 ± 11	0.53
		0.1	0.54 ± 0.12	72 ± 42	0.54
DRV	4.7	1	12.41 ± 1.85	> 1000	1
		0.5	5.19 ± 0.86	> 1000	0.99
		0.1	1.16 ± 0.13	> 1000	0.81
	7.4	1	8.18 ± 0.61	> 1000	1
		0.5	9.14 ± 0.76	> 1000	0.97
		0.1	1.95 ± 0.61	> 1000	0.93
	9.2	1	4.45 ± 0.14	> 1000	0.96
		0.5	3.35 ± 0.13	> 1000	0.87
		0.1	0.90 ± 0.06	> 1000	1

EXT, extrusion; INJ, ethanol injection; DRV, dehydration-rehydration of vesicles.

tively large amounts as 12.4 mmol of drug per mol of lipid at pH 4.7 and an initial drug/lipid ratio of 100 mmol/mol.

In order to determine the effect of the three variables studied (method, pH and drug concentration) on the parameter that quantifies the degree of entrapment, namely, the encapsulation efficiency, such variables were evaluated by multifactor analysis of variance and Table 4 was obtained from the 81 data collected. At a confidence level of 95%, the results showed that the method of liposome obtention was the variable associated with the most important effect (F ratio = 113.098), although drug concentration and pH also exerted some influence. On the other hand, the two-factor interactions are significant, mainly between method and concentration and between method and pH.

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 anticancer drugs has been intensively studied. However, no studies have been reported with thioguanine, a drug used in the treatment of acute or chronic leukemia.

In this paper, we describe the encapsulation of thioguanine in three different kinds of liposomes under different conditions of pH and concentration. A very important factor that determines the encapsulation characteristics of a drug is its chemical structure. The C-N bond in thioguanine has some double bond character; this can be explained by resonance and results in a free base potentially tautomeric in the five-membered ring as well as in the aromatic one. Thioguanine possess five sites that can act as donors or acceptors of protons, and, hence, thioguanine behaves as an acid or as a base as a function of the pH.

From partition coefficients measurements, it can be inferred that the molecule achieves maximal neutrality at pH 4.7 and, for this reason, it is logical to think that species with no net charge (neutral and zwitterionic) predominate at such pH. On the other hand, spectrophotometric measurement affords a pK_a of 8.26. From the molecular structure, it can be inferred that thioguanine is capable of donating two protons or accepting one. However, we have not been able to demonstrate other dissociation constants, either potentiometrically or conductimetrically. A closely related compound, guanine, presents three pK_a values at 3.0, 9,32 and 12.6 (T'so, 1974), and comparing thioguanine with guanine, the pK_a of 8.26 would represent the pH value where an equilibrium exists between the neutral and negatively monocharged form. Such a negative form arises from the loss of a proton present in a

Table 4
Analysis of variance of encapsulation efficiency as a function of the three variables used at a confidence level of 95%

Source of variation	Sum of squares	DF	Mean square	F ratio	Significance level
Main effects	404.46	6	67.41	61.994	0.0000
Method	245.46	2	122.98	113.098	0.0000
Concentration	97.65	2	48.82	44.901	0.0000
pH	60.85	2	30.43	27.982	0.0000
Two-factor interactions	194.27	12	16.19	14.888	0.0000
Method-concentration	128.19	4	32.05	29.473	0.0000
Method-pH	42.43	4	10.61	9.756	0.0000
Concentration-pH	23.64	4	5.91	5.435	0.0008
Residual	67.4	62	1.09		
Total (corr.)	666.15	80			

nitrogen of the purine ring. Based upon the pK_a obtained, 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 reaches 6%.

Based on encapsulation efficiency data, it can be concluded that the optimal method for encapsulating thioguanine in liposomes is the dehydration-rehydration technique, while it is poorly entrapped in uni-or oligolamellar liposomes. We cannot explain the high encapsulation efficiency in the DRV technique by a purely bilayer association of the drug. In this case, there should not be a difference between the different liposome formulations at a determined pH, because it is a partitioning process of a drug between a lipid phase and a water phase and the encapsulation efficiency is normalized and expressed as mol/lipid mol. The greater degree of entrapment can be explained by the fact that thioguanine presents a chemical structure with a relatively large number of points that can undergo hydrogen bonds and, since this kind of binding is favoured by a nonaqueous medium, these bonds are formed in the freeze-drying process. Comparing the encapsulation features with the physical properties of liposomes and the physicochemical characteristics of thioguanine, we have established that this molecule, like amphiphilic drugs, behaves like a permeant molecule which cannot be retained for a long time in liposomes. This property explains the choice of the mini-column technique, which takes little time, as a method of purification for oligo-and unilamellar liposomes, instead of other more time-consuming techniques, such as dialysis or gel filtration. Otherwise, rapid passage of the drug through the liposome membrane during purification, as a result of the structural properties of thioguanine, would occur. In this way, thioguanine tends to escape from the intraliposomal aqueous phase by means of a diffusion process. Consequently, after the purification step, we measure those molecules that are interacting with the lipid bilayer by means of electrostatic forces (both, lipid and drug, are in zwitterionic form). In this way, multilamellar liposomes contain more molecules of drug in the bilayers than the unilamellar ones because they are auto-associated and this abolishes or diminishes the ability for escaping from the vesicle. Furthermore, the fact that maximal drug entrapment in the liposomes was achieved at pH 4.7, i.e., when the molecule presented maximal neutrality, supports the assessment that thioguanine interacts with the lipid components of liposomes.

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