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Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients

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Studies from this laboratory (Mayer et al. (1986) Biochim. Biophys. Acta 857, 123–126) have shown that doxorubicin can be accumulated into liposomal systems in response to transmembrane pH gradients (inside acidic). Here, detailed characterizations of the drug uptake and retention properties of these systems are performed. It is shown that for egg phosphatidylcholine (EPC) vesicles (mean diameter of 170 nm) exhibiting transmembrane pH gradients (inside acidic) doxorubicin can be sequestered into the interior aqueous compartment to achieve drug trapping effiicencies in excess of 98% and drug-to-lipid ratios of 0.36:1 (mol/mol). Drug-to-lipid ratios as high as 1.7:1 (mol/mol) can be obtained under appropriate conditions. Lower drug-to-lipid ratios are required to achieve trapping efficiencies in excess of 98% for smaller (≤ 100 nm) systems. Doxorubicin trapping efficiencies and uptake capacities are related ito maintenance of the transmembrane pH gradient during encapsulation as well as the interaction between doxorubicin and entrapped citrate. This citrate–doxorubicin interaction increases drug uptake levels above those predicted by the Henderson-Hasselbach relationship. Increased drug-to-lipid ratios and trapping efficiencies are observed for higher interior buffering capacities. Retention of a large transmembrane pH gradient (greater than 2 units) after entrapment reduces the rate of drug leakage from the liposomes. For example, EPC/cholesterol (55:45, mol/mol) liposomal doxorubicin systems can be achieved which released less than 5% of encapsulated doxorubicin (drug-to-lipid molar ratio = 0.33:1) over 24 h at 37°C. This pH gradient-dependent encapsulation technique is extremely versatile, and well characterized liposomal doxorubicin preparations can be generated to exhibit a wide range of properties such as vesicle size, lipid composition, drug-to-lipid ratio and drug release kinetics. This entrapment procedure therefore appears well suited for use in therapeutic applications. Finally, a rapid colorimetric test for determining the amount of unencapsulated doxorubicin in liposomal systems is described.

Introduction

Numerous studies have shown that liposomal encapsulation of doxorubicin can provide a significant therapeutic benefit by decreasing dose limiting toxic side effects such as cardiotoxicity [1–9] and nephrotoxicity [7,10] while maintaining or, in some instances, increasing antitumour potency. This decrease in toxicity has been shown to allow higher doxorubicin doses to be administered, resulting in enhanced antitumour efficacy as evidenced in numerous animal models [3,4,8–16]. Although such studies suggest a therapeutic potential for liposomal doxorubicin, previous formulations suffer significant problems with respect to flexibility and stability. These systems exhibit a variety of sizes, drug-to-lipid ratios and lipid compositions which make it difficult to select optimal formulations. The inability to independently vary these parameters in traditional entrapment techniques has precluded the direct correlation of liposome physical characteristics with in vivo toxicity or efficacy behaviour. Further, such formulations often contain labile lipids such as cardiolipin which mitigate against long-term stability.

We have demonstrated that doxorubicin can be accumulated into large unilamellar vesicles which exhibit a transmembrane pH gradient (inside acidic; Refs. 17,18).

Abbreviations: MLV, multilamellar vesicle; LUV, large unilamellar vesicle; EPC, egg phosphatidylcholine; DOX, doxorubicin.

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Trapping efficiencies approaching 100% and excellent drug retention are obtained in a manner which is relatively independent of vesicle size and lipid composition. This has allowed the systematic variation of liposomal doxorubicin formulations with respect to size, drug-to-lipid ratio and lipid composition, and has led to increased understanding of the effects that these liposome characteristics have on in vivo toxicity and antitumour efficacy [18].

Further manipulations of the physical characteristics of liposomal doxorubicin will likely lead to formulations with improved therapeutic activity. For this to occur, a comprehensive assessment of the entrapment procedure and the resulting preparations is necessary. In this report an in-depth characterization of the transmembrane pH gradient-dependent doxorubicin encapsulation procedure is performed. Factors which affect drug trapping efficiency and retention are identified. These studies define conditions required to achieve stable liposomal doxorubicin preparations for vesicle systems exhibiting high drug-to-lipid ratios and small sizes. In addition, the results provide a basic understanding of the mechanism of the uptake process. A simple method for rapidly determining the proportion of free doxorubicin in liposomal preparations is also described.

Materials and Methods

Materials

Egg yolk phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabama, AL, U.S.A.) and was greater than 99% pure as indicated by thin-layer chromatography. Doxorubicin was purchased from Adria Laboratories (Mississauga, Canada). Cholesterol, citric acid, Na_2CO_3 and NaOH were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Methods

Liposomal preparation. Vesicles were prepared by hydrating a lipid film in the presence of the indicated concentrations of citric acid (pH 4.0) to achieve lipid concentrations of 100 mg total lipid/ml. In cases where decreased concentrations of citrate were employed NaCl was included in the buffer solution to achieve isoosmolarity in the higher concentration citrate buffers. The MLVs were then frozen and thawed five times as described previously to achieve equilibrium transmembrane solute distributions [19] and extruded ten times through polycarbonate filters of the indicated pore size [20,21] employing a liposome extruder obtained from Lipex Biomembranes (Vancouver, Canada). Vesicle size distributions were determined by quasielastic light scattering (QELS) employing a Nicomp model 270 particle sizer (Pacific Scientific). Vesicle morphology and size were monitored by freeze-fracture electron microscopy employing a Balzers BAF 400D freeze-fracture apparatus and a Phillips 400 electron microscope as described previously [20,21].

Doxorubicin encapsulation. Transmembrane pH gradients were established by titrating the exterior pH to 7.5 with sodium carbonate or alternatively passing the vesicles over a Sephadex G-50 column equilibrated in 20 mM Hepes, 150 mM NaCl (pH 7.5). Samples were then diluted to the indicated lipid concentrations with 20 mM Hepes, 150 mM NaCl (pH 7.5). Doxorubicin was then added from a stock saline solution and incubated with the vesicles at 60 °C for 10 min with intermittent vortex mixing. The 60 °C incubation is necessary for rapid and complete entrapment of doxorubicin inside vesicle systems containing cholesterol [18]. Thin-layer chromatography and HPLC analysis indicated no degradation of doxorubicin under these conditions.

Vesicle-entrapped doxorubicin was determined by column chromatography. A small aliquot of the liposomal doxorubicin solution was diluted (as necessary) with unbuffered saline to achieve 5 mM lipid concentrations. Phospholipid and doxorubicin concentrations were determined by phosphorus analysis and absorbance at 480 nm, respectively, as described previously [22]. Untrapped doxorubicin was separated from the liposomes by passing 150 μl of the diluted sample over a 1 ml Sephadex G-50 column equilibrated in unbuffered saline. The vesicle-containing eluant was then assayed for doxorubicin and phospholipid. Trapping efficiencies were calculated as the doxorubicin/phospholipid ratio after separation of free drug divided by the doxorubicin/phospholipid ratio before separation.

Free and encapsulated doxorubicin in liposomal systems were determined spectrophotometrically employing a Shimadzu UV-160 spectrophotometer. This procedure utilizes the pronounced change in absorbance (absorbance maxima 480 nm, pH 7.5; 550 and 592 nm, pH 10.5) observed on increasing the pH of doxorubicin solutions from 7.5 to 10.5. This can be followed visually by a colour change from red-orange to blue. The liposomal doxorubicin samples were diluted with 20 mM Hepes, 150 mM NaCl (pH 7.5) to achieve doxorubicin concentrations between 0.05 and 0.10 mM. For each preparation the following sequence of measurements were made. First, the absorbance at 600 nm of the diluted sample was adjusted to zero. Second, the pH of the sample was increased to 10.5 with 1.0 M NaOH (0.02 ml/1.0 ml of sample) and the absorbance at 600 nm was recorded within 2 min. Third, the spectrophotometer was zeroed against a 0.2% Triton X-100 solution. Fourth, the absorbance at 600 nm of the liposomal doxorubicin sample to which Triton X-100 had been added (0.01 ml 20% Triton/1.0 ml of sample) was determined. Free/total doxorubicin ratios were

calculated as the absorbance at 600 nm upon NaOH addition divided by the absorbance after Triton X-100 addition.

In vitro stability of liposome encapsulated doxorubicin was monitored by dialyzing samples (2 mM lipid) for 24 h against 1000 volumes of 20 mM Hepes, 150 mM NaCl (pH 7.5) at 37 °C. At the indicated times, 150-μl aliquots were removed and entrapped doxorubicin was determined employing column chromatography as described above.

Transmembrane pH gradients were monitored employing [^{14}C]methylamine as described previously [23]. Briefly, [^{14}C]methylamine (0.5 μCi/ml) was added to a liposome solution containing < 10 mg lipid/ml. After 15 min, 150 μl aliquots were passed down 1 ml Sephadex G-50 columns at 21 °C equilibrated in 20 mM Hepes, 150 mM NaCl (pH 7.5) to remove unencapsulated methylamine (column run time of 3 to 5 min). Lipid and methylamine concentrations before and after column chromatography were determined by phosphorous assay and scintillation counting, respectively. The transmembrane pH gradient was then calculated according to the relationship:

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{[\text{methylamine}]_{in}}{[\text{methylamine}]_{out}}$$

This procedure has been shown to be an accurate and rapid method for monitoring transmembrane pH gradients [24] and compares very well with results obtained employing ^{31}P-NMR [24] and equilibrium dialysis (data not shown). It should be noted that under the conditions employed the exterior pH did not change during the uptake process.

Results

It is commonly assumed that lipophilic amines permeate membranes in the neutral (deprotonated) from [23,25]. Thus uptake in response to ΔpH relies on permeation of the neutral species and subsequent reprotonation in the acidic internal medium which depletes the internal proton pool. The resulting dependence for doxorubicin on the internal buffer capacity was studied by monitoring drug accumulation while varying the concentration of citric acid inside the liposomes (Fig. 1). For an initial drug-to-lipid ratio of 0.36 : 1 (mol/mol), increasing the citrate concentration from 10 mM to 100 mM produces an increase in the doxorubicin trapping efficiency from 24% to greater than 98%. Further increases in the entrapped citrate concentration above 100 mM result in trapping efficiencies of approx. 100%. It should be noted that alterations in the citrate concentration did not affect the vesicle aqueous trapped volume as determined utilizing [^{14}C]citric acid as an aqueous marker (results not shown).

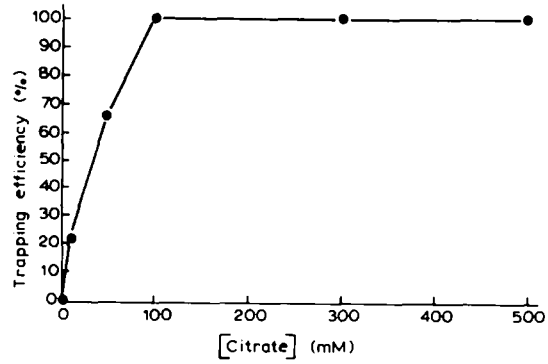

Fig. 1. Effect of internal buffering capacity on doxorubicin uptake into EPC/cholesterol liposomes (mean diameter, 175 nm). Vesicles were prepared from FATMLV precursors in the presence of the indicated citric acid concentrations (pH 4.0). Vesicles (15 mM lipid) were incubated in the presence of doxorubicin (5 mM) for 5 min at 60 °C after the vesicle external medium was brought to pH 7.8. Trapping efficiencies were determined employing column chromatography as described in Materials and Methods.

Doxorubicin entrapment characteristics are also sensitive to the drug-to-lipid ratio of the initial incubation mixture. Sufficiently high levels of accumulated drug may be expected to deplete the interior buffering capacity and collpse the transmembrane pH gradient, thereby inhibiting further drug uptake. This is illustrated Fig. 2, which presents the effect of the initial drug-to-lipid ratio on the level of doxorubicin encapsulation, trapping efficiency and the pH gradient remaining after uptake. Varying the drug-to-lipid ratio for vesicles containing 300 mM citrate (pH 4.0) between 0.11 : 1 and 0.36 : 1 (mol/mol) has no effect on doxorubicin trapping efficiency and values of approx. 100% are achieved in this range (Fig. 2A). Increasing the initial drug-to-lipid molar ratio above 0.36 : 1 results in uptake levels as high as 1.2 mol/mol lipid (Fig. 2B). Trapping efficiencies decrease significantly, however, as the initial drug to lipid ratio is increased above 0.55 : 1 (mol/mol, Fig. 2A). The reason for decreased trapping efficiencies observed for the high drug-to-lipid ratios can be seen in Fig. 2C where the transmembrane pH gradient remaining after doxorubicin encapsulation is monitored as a function of the initial drug-to-lipid ratio. Systems exhibiting drug-to-lipid ratios below 0.36 : 1 (mol/mol) maintain transmembrane pH gradients in excess of 2.0. Doxorubicin entrapment does deplete the internal H^+ pool for all systems studied yielding ΔpH values which are lower than initially imposed. This effect becomes most pronounced for initial drug-to-lipid ratios greater than 0.55 : 1 where the residual ΔpH falls below 1.5 and corresponding trapping efficiencies decrease below 90% (Fig. 2A).

The in vivo toxicity of doxorubicin encapsulated inside EPC/cholesterol vesicles decreases with increasing drug-to-lipid ratio [18]. It is therefore of interest to investigate the ability to achieve high trapping efficien-

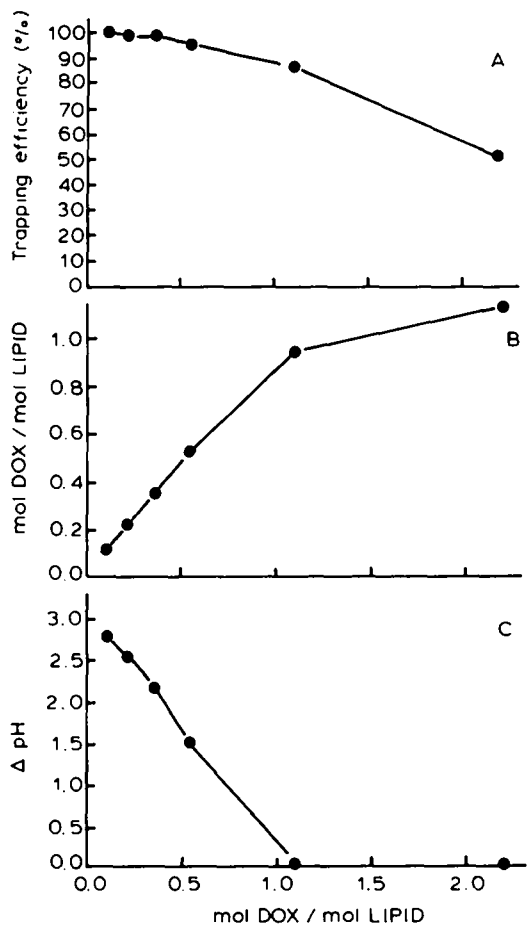

Fig. 2. Effect of drug to lipid ratio on doxorubicin trapping efficiency (A), entrapped drug to lipid ratio (B) and residual transmembrane pH gradient (C). EPC/cholesterol 175 nm vesicles (300 mM citric acid, pH 4.0 inside; pH 7.8 outside) were incubated (5 mM lipid) at 60 °C for 5 min in the presence of doxorubicin at the indicated drug to lipid ratios. The amounts of entrapped drug as well as trapping efficiencies were determined by column chromatography as described in Materials and Methods. Transmembrane pH gradients were determined subsequent to doxorubicin uptake by monitoring the transmembrane distribution of [^{14}C]methylamine (0.5 lCi/ml) and correlating inside/outside ratios to transmembrane ΔpH as described in Materials and Methods.

cies and large residual pH gradients for liposomal systems with uptake levels in excess of 0.33 mol/mol lipid. Fig. 3 demonstrates that entrapped doxorubicin levels can be increased to 1.7 mol/mol lipid (initial drug-to-lipid molar ratio of 2.2 : 1) by increasing the citrate concentration inside the liposomes from 300 mM to 500 mM. Such accumulation levels reflect an apparent interior doxorubicin concentration of 1.6 M for the vesicle systems employed. Furthermore, trapping efficiencies and residual pH gradients of > 95% and 1.3, respectively, are obtained for an initial drug-to-lipid ratio of 1.1 : 1. These results are consistent with the results presented in Fig. 1. However, increasing the entrapped citrate concentration to 1.0 M or decreasing the pH of the encapsulated aqueous medium did not significantly alter the entrapment characteristics over those obtained with 500 mM citrate (data not shown). This may be due to the decreased effective buffering capacity arising from activity coefficient effects or alternatively leakage due to osmotic forces experienced at these high citrate concentrations.

Liposome size is an important additional variable. The ability to generate small (< 100 nm) vesicle systems containing high levels of entrapped doxorubicin may be very desirable in view of the increase in circulation longevity [26] and antitumour activity [18] observed as vesicle size is decreased. Fig. 4 presents the percentage trapping efficiency and residual gradient pH (ΔpH remaining after drug uptake) as a function of the initial doxorubicin-to-lipid ratio for EPC/cholesterol vesicles sized through 200 nm, 100 nm, 50 nm and 30 nm pore size filters. The measured mean diameters of these systems are 175 nm, 98 nm, 65 nm and 55 nm, respectively. Decreasing the vesicle size from 175 nm to 55 nm decreases the doxorubicin trapping efficiency at a drug-to-lipid ratio of 0.36 : 1 from approx. 100% to 63% (Fig. 4A). A more dramatic difference is revealed in the residual pH gradient where a value of 2.2 is observed for the 175 nm system while vesicles exhibiting a mean

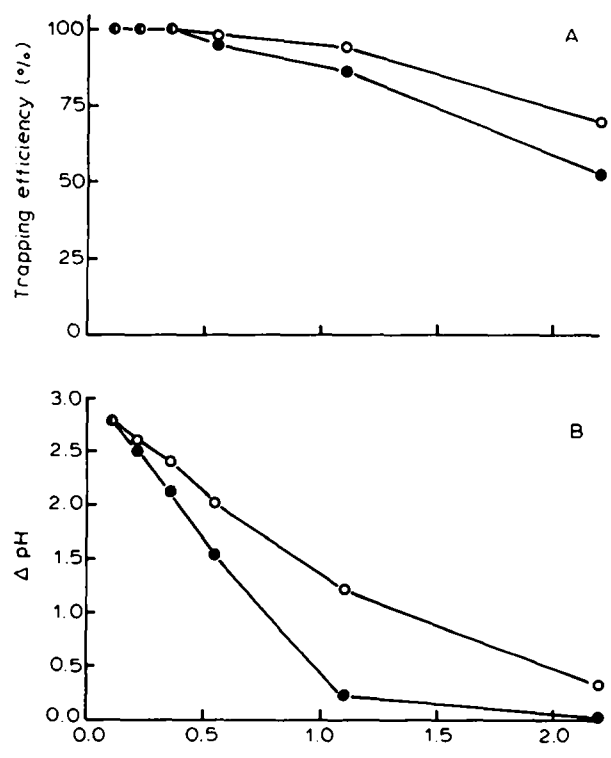

Fig. 3. Percent doxorubicin trapping efficiency (A) and residual transmembrane pH gradient (B) for 175 nm EPC/cholesterol vesicles (5 mM lipid) containing 300 mM (●) or 500 mM (○) entrapped citrate buffer (pH 4.0) as a function of the initial drug-to-lipid ratio. Trapping efficiencies and pH gradients were determined as described in the legend of Fig. 2.

diameter of less than 100 nm retain a ΔpH of less than 0.5 units (Fig. 4B). At a drug-to-lipid ratio of 0.22 : 1, trapping efficiencies in excess of 90% are obtained for all vesicle systems studied. However, residual pH gradients are seen to derease with decreasing vesicle size (Fig. 4B). It should be noted that residual pH gradients for the 65 nm and 55 nm vesicle systems can be increased to more than 2 units at a drug-to-lipid ratio of 0.22 : 1 by increasing the entrapped citrate concentration above 300 mM (data not shown). These results again demonstrate the importance of the buffering capacity of the vesicle interior for pH gradient-dependent doxorubicin encapsulation since decreasing vesicle size decreases the aqueous trapped volume.

The very high entrapped doxorubicin levels achieved during pH gradient-dependent uptake represent potential interior vesicle drug concentrations in excess of 1 M. Studies were undertaken to determine the solubility of doxorubicin in citrate buffer as a function of pH in order to assess whether such concentrations are attainable. As shown in Fig. 5, doxorubicin displays very limited solubility in 300 mM citrate buffer over the

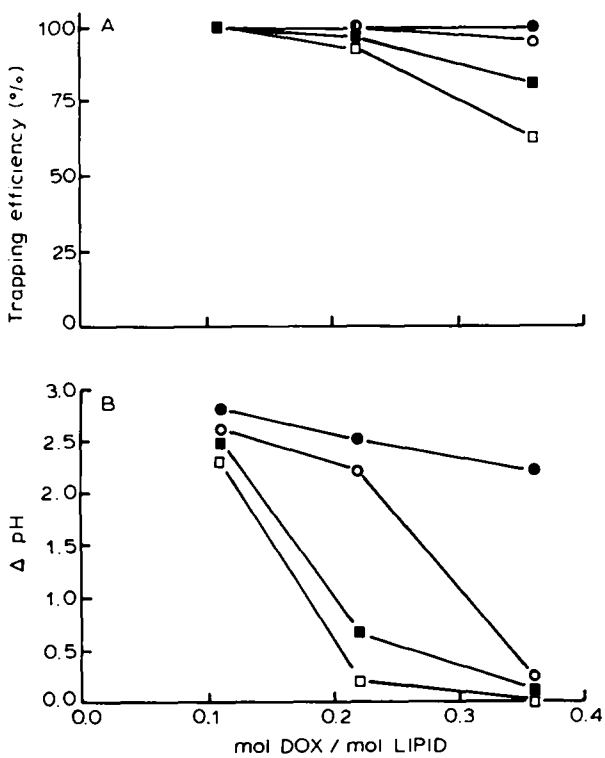

Fig. 4. Percent doxorubicin trapping efficiency and residual transmembrane pH gradient for EPC/cholesterol vesicles obtained by extrusion through 200 nm (●), 100 nm (○), 50 nm (■) and 30 nm (□) pore size polycarbonate filters. The mean diameters of these systems were 175 nm, 98 nm, 65 nm and 55 nm, respectively. All vesicles were prepared in the presence of 300 mM citrate buffer (pH 4.0). The indicated drug-to-lipid ratios reflect initial drug-to-lipid ratios in the incubation mixture (lipid concentration, 5 mM). Percent trapping efficiencies and residual pH gradients were determined as described in the legend of Fig. 2.

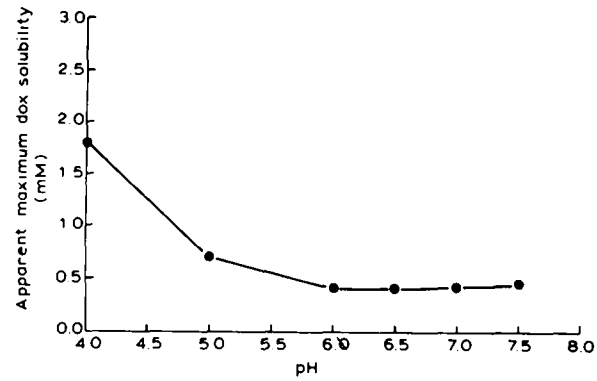

Fig. 5. Solubility of doxorubicin in 300 mM citric acid buffer as a function of pH. Doxorubicin was added to the buffers at an amount sufficient to yield a 20 mM solution if fully dissolved. The samples were heated for 10 min at 60 °C and then cooled to room temperature. Doxorubicin concentrations were determined spectrophotometrically (absorbance at 480 nm) as described in Materials and Methods after removing undissolved drug by centrifugation.

entire range of pH employed in these investigations. The apparent maximum drug solubility is ≤ 0.5 mM between pH 6.0 and 7.5 and increases to 1.8 mM at pH 4.0. These results compare favorably with reports on the solubilities of various lipophilic, cationic drugs in citrate buffers [27]. The low solubility of doxorubicin over this pH range, however, is dependent on the buffer used. For example, doxorubicin solutions in excess of 20 mM at pH 7.5 can readily be prepared utilizing 20 mM Hepes, 150 mM NaCl (data not shown).

In addition to decreasing doxorubicin trapping efficiencies, depletion of the ΔpH also reduces the ability of liposomes to retain encapsulated doxorubicin. Fig. 6 shows that less than 5% of the entrapped doxorubicin is released over 24 h at 37 °C for EPC/cholesterol 175 nm systems with initial drug-to-lipid ratios between 0.11 : 1 and 0.36 : 1. Under these conditions (300 mM entrapped citrate) residual ΔpH values were in excess of 2.0 (see Fig. 2C). Liposomes exhibiting initial drug-to-lipid ratios greater than 0.5 : 1 and final ΔpH values less than 2.0 (Fig. 2C) display increased doxorubicin release (Fig. 6). After 24 h, 25%, 51% and 70% of the encapsulated doxorubicin is released for initial drug-to-lipid ratios of 0.55 : 1, 1.1 : 1 and 2.2 : 1, respectively. Similar relationships between doxorubicin retention and the residual pH gradient are observed for vesicle systems exhibiting decreasing concentrations of entrapped citrate at a fixed drug-to-lipid ratio (data not shown).

In a final area of investigation, studies were undertaken to develop a rapid and simple assay procedure for free and liposome-encapsulated doxorubicin. Fig. 7 shows the absorbance spectra between 400 and 700 nm for doxorubicin at pH 7.5 (a) and pH 10.5 (b). Increasing the pH from 7.5 to 10.5 causes a shift in the absorbance peak wavelength from 480 nm to 550 nm and 592 nm. This pH-dependent spectral response pro-

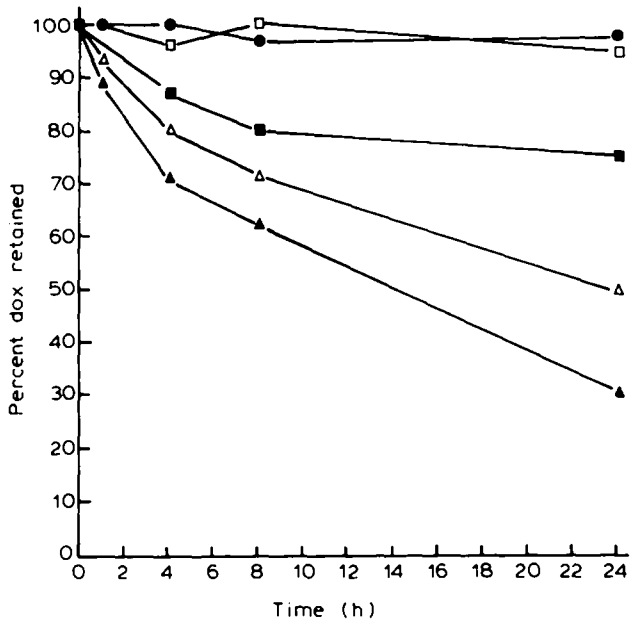

Fig. 6. Release of doxorubicin from EPC/cholesterol (55:45, mol ratio) vesicles under dialysis conditions (20 mM Hepes, 150 mM NaCl, pH 7.5 external buffer) at 37°C. Entrapped doxorubicin was determined by column chromatography as described in Materials and Methods. Samples were obtained as described in the legend to Fig. 2 for initial drug-to-lipid ratios (mol:mol) of 0.11:1 (●), 0.22:1 (○), 0.36:1 (□), 0.55:1 (■), 1.1:1 (△) and 2.2:1 (▲).

vides the basis for determining free and vesicle entrapped doxorubicin in liposomal preparations. In particular, raising the pH of the liposomal doxorubicin solution to 10.5 will result in a spectral shift of free doxorubicin but not vesicle encapsulated drug. The resulting difference in absorbance before and after Triton X-100 addition (which exposes all doxorubicin to the alkaline medium) at 600 nm therefore reflects the relative amount of untrapped doxorubicin in the preparation.

This spectroscopic analysis of drug entrapment in liposomal doxorubicin preparations was compared with column chromatography methods which directly mea-

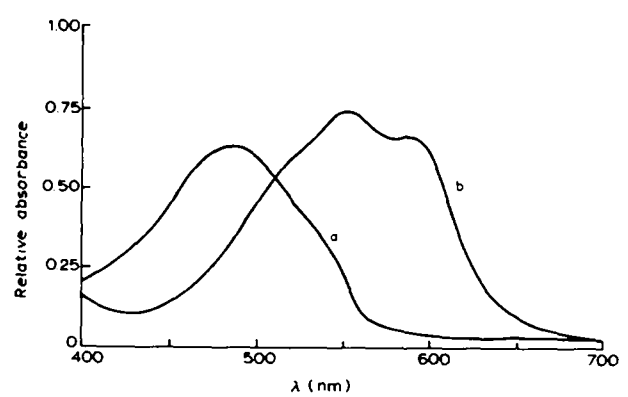

Fig. 7. Absorbance spectra between 400 and 700 nm for doxorubicin solutions adjusted to pH 7.5 (a) and 10.5 (b).

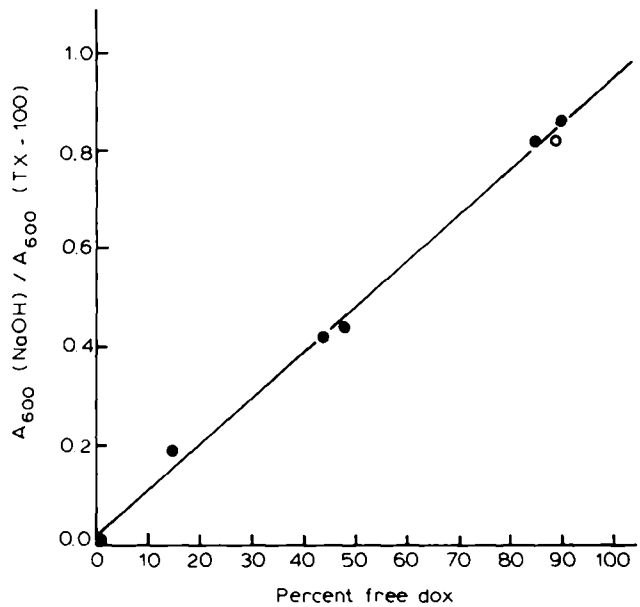

Fig. 8. Comparison of free/total doxorubicin ratios with the absorbance ratio at 600 nm before and after addition of Triton X-100 to alkalinized liposomal doxorubicin. Doxorubicin was actively encapsulated (closed symbol) employing 10 mM lipid (EPC/cholesterol, 55:45, mol ratio) and 2 mM drug while passive entrapment (open symbol) utilized 50 mM lipid (EPC) and 2 mM drug as described in Materials and Methods. Spectroscopic analysis and quantitation of free and total doxorubicin by column chromatography was completed as described in Materials and Methods. The line represents a linear least-squares analysis fit to the data.

sure free and vesicle associated drug to correlate the absorbance ratio values to actual free doxorubicin/total doxorubicin ratios over a wide range of trapping efficiencies. EPC/cholesterol vesicles exhibiting pH gradients (acidic interior) of varying magnitude were utilized to construct liposome systems with trapping efficiencies ranging from 10 to 99%. Fig. 8 demonstrates that the absorance ratio at 600 nm described here accurately reflects the ratio of free/total doxorubicin in the vesicle preparations. The spectroscopic analysis method was also applied to EPC vesicles in which doxorubicin had been passively trapped during vesicle formation to ensure that these results were not specific to liposomal doxorubicin obtained by active entrapment. Fig. 8 (open symbol) shows that the absorbance ratio at 600 nm for this sample correlates well with the free/total doxorubicin value determined by column chromatography. The doxorubicin trapping efficiency of 13% obtained for this preparation compares well with trapping efficiencies observed previously for aqueous markers employing similar vesicle systems and conditions [21]. An additional benefit of the absorbance characteristics of the spectral shift is that it allows the relative amount of free doxorubicin in liposome preparations to be assessed visually. Although such an analysis is clearly qualitative, the occurrence of 5% free drug can be detected and a dramatic colour change (red

to blue) is observed for systems exhibiting greater than 15% free drug (results not shown).

Discussion

The studies presented here demonstrate that the uptake of doxorubicin into LUVs in response to transmembrane pH gradients represents a flexible technology, allowing efficient drug entrapment for a variety of liposome sizes and drug-to-lipid ratios. Here we discuss the mechanisms involved in the accumulation process and the utility of the resulting systems in pharmaceutical applications.

The ability of liposomes to accumulate doxorubicin in response to a transmembrane pH gradient (inside acidic) is similar to uptake behaviour demonstrated elsewhere for dibucaine [23] and dopamine [28]. The uptake process involves transmembrane permeation of the neutral form of the drug which is subsequently reprotonated on the vesicle interior leading to transmembrane concentration gradients that are driven by the proton gradient. Thus, in order to achieve high entrapment levels the interior must be highly buffered to maintain the pH gradient during accumulation of drug (which depletes the interior pool of protons). This is in general agreement with the results presented here where high doxorubicin trapping efficiencies, high drug-to-lipid ratios and stable drug retention are closely related to the ability of the liposomes to maintain a significant transmembrane pH gradient (> 2 units, inside acidic).

Although these results suggest a typical Henderson-Hasselbach equilibrium redistribution of a weak base drug across the membrane, a rigorous analysis of the present studies reveals a more complex process for pH gradient-dependent doxorubicin accumulation into citrate-containing vesicles. Vesicle properties that lead to rapid depletion of the entrapped buffering capacity such as low trapped volumes, decreased entrapped citrate concentrations and high drug-to-lipid ratios do yield decreased doxorubicin trapping efficiencies and residual pH gradients. However, the transmembrane drug concentration gradients observed for these systems are far greater than predicted by the Henderson-Hasselbach relationship. This is shown in Table I, where apparent $\log[DOX]_{in}/[DOX]_{out}$ values are in excess of 3 for systems exhibiting measured residual pH gradients ranging from 0.1 to 2.3 units. Corresponding interior doxorubicin concentrations (assuming that all of the drug is freely soluble in the entrapped aqueous space) vary between 327 mM and 950 mM. In view of the relatively low solubility of doxorubicin in citrate buffer over the pH range studied here (Fig. 5 and Ref. 27) it is likely that at these concentrations doxorubicin is present in a precipitated or stacked form. Anthracyclines have been shown to adopt such structures at high concentrations [29].

The above interpretation predicts that doxorubicin uptake is dictated by the equilibration of entrapped drug between soluble and insoluble forms as well as the proton gradient-driven transmembrane distribution of soluble doxorubicin. Therefore, although the apparent $[DOX]_{in}/[DOX]_{out}$ (column 7 of Table I) may be far in excess of the residual ΔpH (column 8 of Table I), the true (soluble) transmembrane concentration gradient of doxorubicin may, in fact, correspond to the proton gradient. If this is indeed the case, then the interior soluble doxorubicin calculated according to the rela-

TABLE I

Summary of characteristics for doxorubicin uptake into various vesicle systems

Mean diameter	Lipid concn. (mM)	Entrapped [citrate] (mM)	Drug/lipid [a] (mol/mol)	Trapped volume (μl/μmol lipid)	Trapping efficiency (%) [b]	Apparent $\log\frac{[Dox]_{in}}{[Dox]_{out}}$ [c]	ΔpH [d]	Theoretical [e] soluble $[Dox]_{in}$ (mM)
175 nm	5	300	0.36:1	1.1	≥ 98	3.96	2.2	5.7
98 nm	5	300	0.22:1	0.65	98	3.96	2.3	4.4
55 nm	5	300	0.22:1	0.53	95	3.85	0.7	0.3
55 nm	5	300	0.22:1	0.35	91	3.76	0.2	0.2
175 nm	5	300	1.1:1	1.1	88	3.15	0.1	0.8
	5	500	1.1:1	1.1	95	3.64	1.3	5.5
	5	300	0.55:1	1.1	96	3.58	1.5	3.5
	0.1	300	0.55:1	1.1	29	3.68	1.3	0.8

[a] Drug-to-lipid ratios reflect initial incubation conditions.

[b] Trapping efficiencies were determined as described in Methods.

[c] Apparent interior doxorubicin concentrations were calculated assuming all of the drug is soluble. In cases where trapping efficiencies $\geq 98\%$, exterior drug concentrations were assumed to be 2% of the total concentration.

[d] The values represent pH gradients measured subsequent to doxorubicin uptake.

[e] Theoretical internal doxorubicin concentrations were calculated on the basis of the measured ΔpH and the exterior (untrapped) drug concentration after uptake employing the relationship: $[\text{soluble DOX}]_{in}/[DOX]_{out} = [H^+]_{in}/[H^+]_{out}$.

tionship $[\text{soluble DOX}]_{in} = [\text{DOX}]_{out} \times [H^+]_{in}/[H^+]_{out}$ should approximate the maximum apparent solubilities determined experimentally. As shown in column 9 of Table I, theoretical interior soluble doxorubicin concentrations calculated in this manner compare favorably with the measured doxorubicin solubilities in citrate buffers (see Fig. 5) for a wide range of lipid concentrations, entrapped buffering capacities and drug-to-lipid ratios. These values also are orders of magnitude lower than those calculated assuming total drug solubility.

The above analysis suggests that doxorubicin uptake into the vesicle systems utilized in this study proceeds until the exterior drug concentration is depleted to the point where $[\text{soluble DOX}]_{in}/[\text{DOX}]_{out} = [H^+]_{in}/[H^+]_{out}$. Thus, under the conditions typically employed here, significant depletion of the external drug pool is required to achieve true transmembrane doxorubicin concentration gradients which reflect the proton gradient, even for relatively small ΔpH values. This is also consistent with the influences of the vesicle trapped volume and entrapped citrate concentration since decreased interior buffering capacities will result in a more rapid dissipation of the ΔpH. Equilibration of the proton and soluble doxorubicin transmembrane concentration gradients will therefore be achieved at lower drug uptake levels. However, it should be noted that since calculated theoretical interior drug conccentrations (Table I) are generally higher than the apparent maximum drug solubilities determined experimentally (Fig. 5), this interpretation may not completely explain the doxorubicin uptake process. Therefore, additional factors such as activity coefficient and partitioning effects may play minor roles in governing doxorubicin uptake in response to transmembrane pH gradients. Whether this analysis is appropriate for systems with significantly lower drug-to-lipid ratios or other buffer compositions is not clear.

The precipitation or stacking model descirbed above is also consistent with the enhanced doxorubicin retention characteristics of pH gradient systems, especially in cases where the pH gradient has been dissipated. Presumably equilibration between soluble and insoluble forms of the drug would limit the rate of doxorubicin release from the vesicles. Clearly, increased retention is related to the magnitude of the transmembrane pH gradient. However, this may be due to the actual pH of the vesicle interior after uptake since elevated pH values would result in increased concentrations of the more membrane permeant unprotonated (neutral) species of doxorubicin.

In addition to providing a basic understanding of the ΔpH-dependent doxorubicin uptake process, the ability to generate well characterized liposomal doxorubicin preparations is of importance for in vivo therapeutic use. A thorough understanding of the factors which dictate entrapment and drug release properties such as described here is necessary to systematically alter individual physical characteristics of liposomal doxorubicin and evaluate their influences on the biological activity of the drug. In this context, liposomal formulations of doxorubicin demonstrate therapeutic advantages related to reduced toxicity and enhanced efficacy. Certain characteristics of the systmes achieved here by encapsulation in response to pH gradients have attractive featured in this regard. First, systems with high drug-to-lipid ratios have been shown to exhibit reduced toxicity (as indicated by LD_{50} studies in rodents). Second, it has been shown that small liposomal doxorubicin systmes exhibit enhanced antitumour efficacy [11,18]. As demonstrated here, 55 nm diameter systems can be readily loaded to achieve trapping efficiencies in excess of 95% and drug-to-lipid ratios of 0.2:1 (mol/mol). Third, these formulations have desirable properties as pharmaceuticals. For instance, the drug can be loaded at the time of use, thus avoiding problems associated with drug retention during prolonged storage. Also, trapping efficiencies approaching 100% can be attained wereas previous entrapment techniques typically yield trapping efficiencies of 50% or less [18,30].

These advantages have led to liposomal doxorubicin formulations which are now undergoing clinical trials. Such clinical applications may require a rapid and straightforward test for liposomal encapsulation of drug. Although several techniques (such as gel filtration chromatography, ion exchange chromatography and dialysis) exist for determining the amount of untrapped doxorubicin in liposomal systems [1,3,4,21,31], these are generally time consuming and require specialized scientific equipment. The colorimetric assay for untrapped doxorubicin described here does not require the use of chromatography equipment and can be completed within minutes as opposed to several hours required for dialysis methods.

In summary, vesicles exhibiting transmembrane pH gradients (inside acidic) can be utilized to encapsulate doxorubicin in a manner where trapping efficiencies, drug-to-lipid ratios and drug retention properties can be reasily manipulated to obtain the desired characteristics. In addition, this encapsulation technique is relatively independent of variables such as lipid composition and size and exhibits pharmaceutically desirable properties. Given that the response of numerous other lipophilic, cationic drugs to transmembrane pH gradients is similar to doxorubicin [27] it is likely that this technology will be useful in developing liposomal delivery systems for a wide variety of therapeutic agents.

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