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Encapsulation of ampicillin in reverse-phase evaporation liposomes: a direct evaluation by derivative spectrophotometry

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Summary

Ampicillin has been entrapped in phospholipid vesicles by reverse-phase evaporation technique. The relationship between lipid composition and the encapsulation efficiency or the release of ampicillin-loaded liposomes was studied in vitro using a derivative spectrophotometry assay. The encapsulation degree was closely dependent on the lipid mixture used in liposome preparation: in particular, phosphatidylcholine (DPPC) vesicles containing cholesterol (CHO) or lipopolysaccharide (LPS) had a lower entrapment efficiency than liposomes prepared with DPPC alone, whereas the presence of cardiolipin (CL) conferred an opposite trend. From the release kinetics it appeared that vesicles leaked the carried drug by a biphasic feature both dialyzing against buffer or in bulk solution. These observations indicate that for the in vivo use of ampicillin-loaded liposomes as chemotherapeutic agents one must pay attention to the lipid composition of the vesicle, in order to modulate the dose–response effect.

Introduction

Liposome encapsulation of a variety of antimicrobial agents has improved therapeutic efficacy, particularly in the treatment of disseminated, facultative, intracellular bacterial infections (Swenson et al., 1988).

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Abbreviations: CHO, cholesterol; CL, cardiolipin; DPPC, l-α-phosphatidylcholine dipalmitoyl; LPS, lipopolysaccharide; REV or REVs, reverse-phase evaporation vesicles.

Liposomes injected intravenously concentrate in organ with sinusoidal capillaries containing reticuloendothelial cells, such as the liver, spleen and bone marrow (Ladigina and Vladimirsky, 1986; Orozco et al., 1986; Popescu et al., 1987), organs which are frequently the sites of localization of disseminated bacterial infections.

Previous studies have demonstrated that liposomal encapsulation of ampicillin results in an increased availability of the antibiotic for the intracellular bacterium *Listeria monocytogenes* in murine macrophages (Bakker-Woundenberg et al., 1985, 1986).

Nowadays, the use of liposomes as drug carrier is becoming of increasing usefulness. In fact, toxicity associated with certain drugs can be reduced

if the agent is presented in association with lipid vesicles (Bally et al., 1990).

Descriptions of use of different types of liposomes are also reported in an in vitro investigation of whether the lipid composition influences the rate of intracellular liposomal degradation and, hence, the rate at which liposome-encapsulated ampicillin is released intracellularly and becomes available to exert its antibacterial effect (Bakker-Woundenberg et al., 1988). Other authors demonstrated, in animal models, that the activity of certain drugs can be modulated by modification of liposomal lipid composition and size (Gabizon et al., 1982; Mayer et al., 1989).

In this paper, using a biologically non-invasive spectrophotometric assay (Di Giulio et al., 1989), we measure the degree of liposome-entrapped ampicillin and its release, since it is known that penicillins freely permeate the lipid bilayer (Hiruma et al., 1984).

Our experiments were set up to study the effect of the different lipidic mixtures upon the ampicillin-loaded vesicle formation, in order to better control the dose–response effect and the release of the entrapped drug.

Materials and Methods

Chemicals

Cholesterol (CHO), dipalmitoyl-1-α-phosphatidylcholine (DPPC), lipopolysaccharide (LPS) and cardiolipin (CL) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ampicillin was obtained from commercial source and was used without further purifications. Chloroform and all other chemicals were from Merck (E. Merck, Darmstadt, Germany) and were reagent grade.

All experiments were carried out in 0.1 M potassium phosphate buffer, pH 7.4, referred to as buffer.

Preparation of reverse-phase evaporation vesicles (REV)

The vesicles were prepared according to Szoka and Papahadjopoulos (1978), with minor modifications, as follows: 50 mg of total lipids at various weight ratios (see Table 1) were dissolved in 5 ml of chloroform and then 5 ml of a solution containing ampicillin 500 μM was added. The resultant two phases system was then sonicated and processed as previously reported (Di Giulio et al., 1989) to obtain the liposome suspension. This suspension was washed three times with buffer to remove non-encapsulated drug and on the third supernatant solution was checked that both the zero-order and the derivative absorption spectra gave zero. Finally, the liposomes were resuspended to a final volume of 1.2 ml in fresh buffer and used for the following studies. The liposome volumes were estimated according to Kinsky et al. (1969).

TABLE 1

Ampicillin encapsulation efficiency of various types of reverse-phase evaporation liposomes

Lipid composition (weight ratio)	Ampicillin encapsulation into liposomes [a] (μM)	Encapsulation (%)
DPPC	224 ± 38	45 ± 8
DPPC:CHO (7:3)	122 ± 10	24 ± 2
DPPC:LPS (7:3)	128 ± 35	26 ± 7
DPPC:CL (7:3)	394 ± 6	79 ± 1
DPPC:CL:LPS (7:2:1)	270 ± 41	54 ± 8

The ampicillin concentration in liposome preparation was 500 μM and referred as 100%.

[a] Each value is expressed as mean ± S.D. of three independent experiments.

Release experiments

The liposome suspension was extensively dialyzed at 4 °C against 1000 volumes of buffer. Aliquots of 200 μl of liposome suspension were drawn at fixed time intervals (from 0 to 48 h) and centrifuged for 15 min, at 10 °C and 30000 × g. After centrifugation, the supernatant was discarded and the pellet resuspended in fresh buffer to reconstitute the original volume. 40 μl of this latter suspension was used to determine the degree of residual entrapped ampicillin. The analyses, in triplicate, were carried out using third derivative spectrophotometry as previously reported (Di Giulio et al., 1989). Alternatively the ampicillin-loaded liposome suspension was kept

at 4 °C in buffer. At fixed intervals, from 0 to 48 h, aliquots were drawn and processed as above. In parallel experiments 5 or 10 mM $MgCl_2$ were added. Values were expressed as percent of residual entrapped drug and referred to 40 μl of the whole liposome suspension taken as 100% of encapsulation.

Measurements and calculations

For the measurements the same technical devices as previously reported (Di Giulio et al., 1989) were used. The measurements were made by fitting the third derivative peak–trough amplitudes expressed in cm of liposome samples into the regression curve of ampicillin concentrations versus peak–trough amplitude, as follows:

$$Y = 0.0259X + 0.05 \qquad (r = 0.999)$$

where Y is the peak–trough amplitude in cm between +233 and −238 nm, X is the ampicillin concentration (ranging in our standard curve between 25 and 500 μM), and r is the correlation coefficient.

Results and Discussion

Reverse-phase evaporation liposomes were adopted to entrap ampicillin following the procedure established by Szoka and Papahadjopoulos (1978, 1980), since this technique is relatively simple, reproducible and produces liposomes mostly unilamellar; under our experimental conditions the vesicle size ranged between 0.05 and 1 μm as measured by electron microscopy images (data not shown). Last, the entrapment efficiency of REVs was usually higher with respect to other types of vesicles, e.g., multilamellar liposomes (Szoka and Papahadjopoulos, 1980; Taylor et al., 1990).

The ampicillin encapsulation of various types of liposomes was calculated by means of deriva-

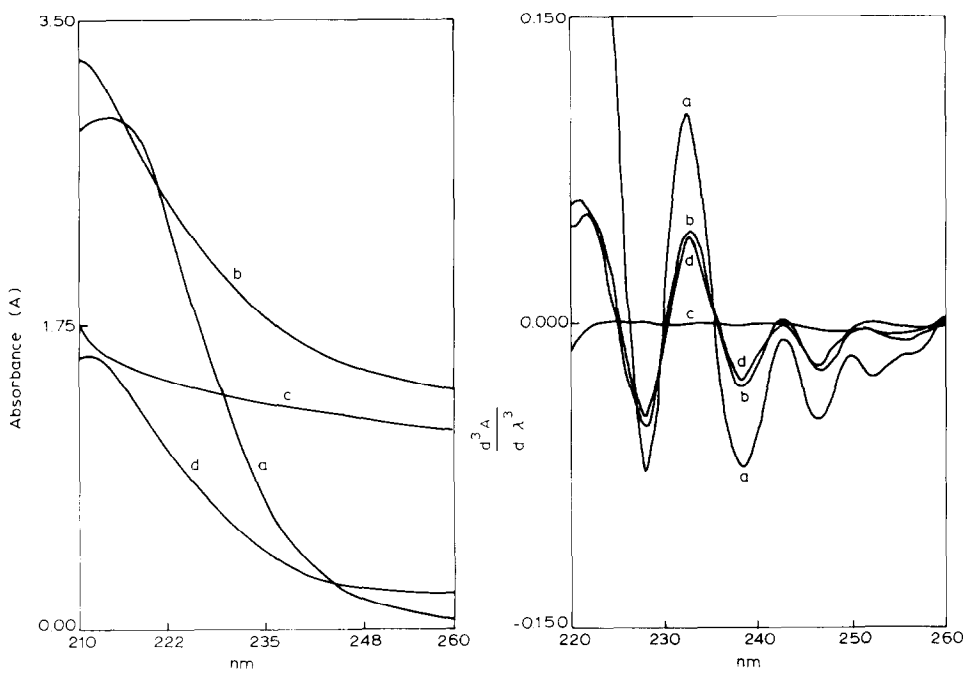

Fig. 1. Zero-order (left) and third-order derivative (right) spectra of ampicillin solution. a, 0.5×10^{-3} M ampicillin in buffer solution; b, ampicillin-loaded DPPC liposomes versus buffer solution in the reference cuvette; c, empty DPPC liposome solution; d, ampicillin-loaded DPPC liposomes versus empty DPPC liposome solution in the reference cuvette.

tive spectroscopy, following the method reported elsewhere (Morelli 1988; Di Giulio et al., 1989). In Fig. 1 the zero- and third-order derivative spectra of ampicillin are shown. The peak–trough amplitude from the third-order spectra allowed to correlate the ampicillin content in liposomes with the peak height, avoiding the troubles due to the sample turbidity. Using this method we calculated the entrapment efficiency of different liposomes prepared from various lipid cocktails with a constant ampicillin concentration of 500 μM in the aqueous phase.

From Table 1 an overall high percentage of entrapped drug can be readily noticed, which ranged in our conditions between 24% and 79% of the initial drug concentration and which was closely correlated to the liposomal lipid composition (Table 1). In particular, vesicles containing 30% (w/w) of cholesterol or lipopolysaccharide had a lower encapsulation efficiency, in comparison to DPPC liposomes; conversely those with cardiolipin, a characteristic phospholipid extracted from the bacterial wall, had the highest capacity to entrap ampicillin. In our system, the addition of CHO to DPPC vesicles reduced the entrapment percentage from about 45% to 24%; the corresponding calculated internal liposome volumes give values of 6.1 and 3.5 μl μmol^{-1} of lipids in DPPC and DPPC:CHO (7:3) liposomes, respectively, which paralleled the entrapment capacity of these vesicles. It should be noted that some differences reported in the literature, for example by Taylor et al. (1990), refer to a different kind of liposomes and to the entrapment of structurally different molecules. Concerning the entrapment effect, another aspect which cannot be in principle excluded is the adsorption of ampicillin on the outer surface and/or in the lipidic bilayer, as we already reported in the case of cefalexin (Di Giulio et al., 1989), which certainly contributes to the total amount of 'entrapped' or anyway carried drug by liposomes.

As far as the release of ampicillin from liposomes is concerned, it also depends on the lipidic composition used; in fact, when the ampicillin-loaded vesicles were either extensively dialysed against buffer or suspended in the bulk solution they leaked the carried β-lactam in different amounts during the experiments (Table 2). The release kinetics seems to be a biphasic process: during the initial phase of rapid drug leakage in the first 4–6 h about 50% of the entrapped drug was released, while a slow release during the following 42–44 h occurred, in which only a further 20% was lost (Figs 2, 3). The first rapid loss of ampicillin could be attributed to the desorption of the drug from the outer shell of liposomes; another alternative or concomitant phenomenon would concern the existence of small

TABLE 2

Release of entrapped ampicillin from different types of liposomes

Type of liposomes (weight ratio)	Moles encapsulated [a]	% of drug released after 48 h	
		A	B
DPPC	40 ± 6	69	39
DPPC:CHO (7:3)	19 ± 2	59	68
DPPC:LPS (7:3)	21 ± 6	72	46
DPPC:CL (7:3)	64 ± 1	75	50
DPPC:CL:LPS (7:2:1)	44 ± 7	80	64

A, release after dialysis against 1000 volumes of buffer; B, release in bulk solution.

[a] Values are expressed as mol ($\times 10^{-8}$)/mg of liposomal lipids.

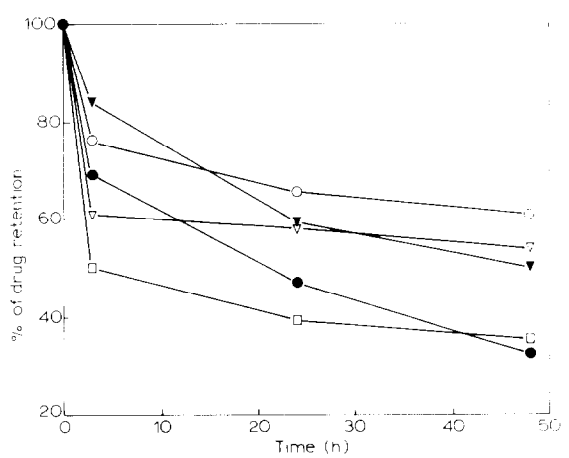

Fig. 2. Time-course of ampicillin release in bulk solution. ○, DPPC; ●, DPPC:CHO (7:3 w/w); △, DPPC:LPS (7:3 w/w); ▲, DPPC:CL (7:3 w/w); □, DPPC:CL:LPS (7:2:1 w/w/w). Each value is the mean of three separate experiments.

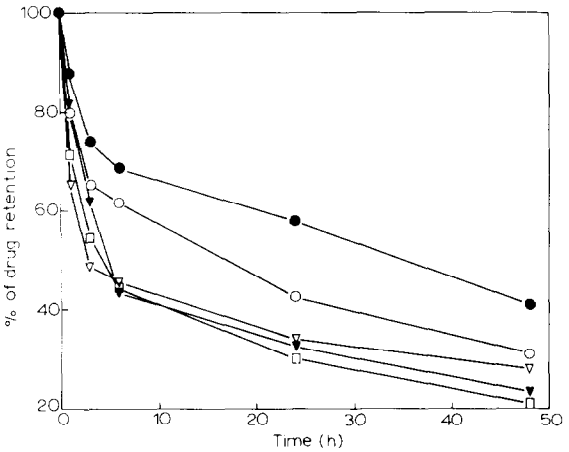

Fig. 3. Time-course of ampicillin release by dialysis. ○, DPPC; ●, DPPC:CHO (7:3 w/w); △, DPPC:LPS (7:3 w/w); ▲, DPPC:CL (7:3 w/w); □, DPPC:CL:LPS (7:2:1 w/w/w). Each value is the mean of three separate experiments.

liposomes, which, as already demonstrated (Taylor et al., 1990), are always present in the REV preparations and, in principle having a higher area/volume ratio than the larger ones, should release the drug quite rapidly. On the other hand, it is difficult to correlate in a more rigorous manner the leakage of ampicillin with all the parameters involved, since most of them are not yet well understood or described. From our data it appears that the presence in the lipidic shell of liposome of cardiolipin or lipopolysaccharide confers intermediate features in release kinetics.

Starting from the observation of Hiruma et al. (1984), who found a specific permeability decrease of cephalosporins due to Mg^{2+} ions in LPS-containing liposomes, we studied the influence of Mg^{2+} on the release of ampicillin by DPPC-LPS liposomes. In our conditions the clearance of this β-lactam from liposomes was independent of the presence of 5 or 10 mM $MgCl_2$ (data not shown).

From these data, the reverse-phase evaporation liposomes appear to be a suitable tool to carry significant levels of ampicillin and to release it in a time lag which could be useful for a therapeutic use; among all the lipids tested, cardiolipin seems to be the most promising one, not only for the properties here reported, but also because this type of liposome should represent a more powerful chemotherapeutic agent by the fusion with bacteria.

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