Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 10, pp. 1159–1164, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00
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The ultraviolet derivative spectrophotometric determination of neutral liposome-entrapped β-lactam antibiotics

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Abstract: A simple, non-invasive spectrophotometric assay to measure the concentration of some β-lactam antibiotics in turbid solutions containing liposomes was carried out. Since zero-order spectra gave strong interference because of sample turbidity, derivative spectrophotometry was used to enhance the spectral details. Derivative spectra showed bands in the ultraviolet region due to the presence of the cephalosporin and penicillin β-lactams. A linear relationship between derivative amplitudes and antibiotic concentration was found when antibiotic-containing liposome solutions were measured. A saturative trend in the liposome-encapsulation was observed. The antibiotic entrapment was lowered by increasing the cholesterol–phospholipid ratio in the mixture used for liposome preparation. After treatment of antibiotic-loaded liposomes with β-lactamase, a hydrolytic enzyme specific for β-lactams, the remaining antibiotic concentration decreased significantly, showing that some of the antibiotic was retained on the outer surface of the vesicles.

Keywords: *Liposome vesicles; β-lactam antibiotics; derivative spectrophotometry.*

Introduction

Liposomes are largely used as biological carriers for drugs [1, 2], enzymes [3–6], and other molecules. Recently, many authors have shown that the entrapment of antibiotics into liposomes [7, 8] confers a significant degree of protection against the enzymic hydrolysis or inactivation of the drug by bacterial enzymes, thus enhancing its antimicrobial activity [9]. In addition, most free antibiotics cannot permeate the cell membrane and consequently are ineffective when the bacteria are proliferating within the cells. Therapy, using drugs that are encapsulated in liposomes may provide an efficient countermeasure [10].

Several techniques are used to measure the degree of solute entrapment, e.g. using radiolabelled [11] or fluorescent materials [12], or rupture of liposome and subsequent measurements of the dissolved material. The encapsulation efficiency was also indirectly

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calculated from the sedimentation coefficient of loaded liposomes and by measurement of the diffusion constant of the entrapped materials [13]. Of these methods, direct spectrophotometry readings have little value because of the turbidity of the liposome solutions. Derivative spectrophotometry has been used as a convenient approach to the problem of resolving spectral overlap in pharmaceutical analysis [14, 15], in analytical biochemistry [16, 17] and, more recently, for the determination of mixtures of different cephalosporins [18]. It has also been used to solve the problem of sample turbidity and matrix background and to enhance spectral details [19]. For example, Gaber *et al.* used derivative spectrophotometry to demonstrate the encapsulation of haemoglobin in vesicles and to calculate the trapping efficiency [20].

The aim of this work was to develop a simple and biologically non-invasive method for the analysis of the β-lactam antibiotics vehiculated by liposomes using second (cephalosporins) or third (penicillins) derivative spectrophotometry.

Materials and Methods

Lipids, antibiotics and other chemicals

Cholesterol (CHO), dipalmitoyl l-α-phosphatidylcholine (DPPC) and cephaloridine were purchased from Sigma Chemical Co. (St. Louis, MO, USA); ceftriaxone, cephalexin, ampicillin and carbenicillin were from commercial sources and were used without further purifications. Chloroform, sodium cholate and all other chemicals were from E. Merck (Darmstadt, FRG) and were reagent grade.

All experiments were carried out in 0.1 M potassium phosphate buffer, pH 7.4, unless otherwise stated.

Preparation of reversed-phase evaporation vesicles (REV)

The liposomes were prepared according to Szoka and Papahadjopoulos [21] with minor modifications, as follows: 50 μmol of lipids (DPPC + CHO, at different molar ratios) were dissolved in 5 ml of chloroform and the aqueous phase, 5 ml of buffer (for preparing empty liposomes) or 5 ml of buffer containing various amounts of the antibiotic to be tested, were added. The resultant two phase system was briefly sonicated (3 min, alternating 30 s sonication and 30 s stand-by periods) in an exponential tip sonicator (Lab. Line Instr., Melrose, IL, USA), until the mixture became a homogeneous opalescent dispersion. The mixture was then placed on a rotary evaporator and the organic solvent removed under reduced pressure (water aspiration) at 30°C for 3 h. Then the preparations were centrifuged 30 min at 12,000*g* to remove non-encapsulated antibiotics. Finally, the liposome pellet was washed three times with buffer. It was confirmed that both the zero-order and the derivative absorption spectra of the third supernatant solution were zero. The liposome pellet was diluted with buffer to the final volume of 1.2 ml and used for spectrophotometric measurements.

Spectrophotometric apparatus and measurements

A UVIKON 860 double beam UV–vis spectrophotometer (Kontron Instr. AG, Switzerland), with 1-cm quartz cells was used. Suitable settings were: bandwidth, 2 nm; response time, 1 s; scan speed, 50 nm min^{-1}. The recorder scale expansion was also optimized to facilitate readings on the recorder tracing. The derivative spectrophotometric measurement which was used to prepare analytical working curves is the so-called "peak–trough amplitude" between a characteristic maximum and a minimum, as

reported by Morelli [18]. The best correlation between peak–trough amplitude and concentration are the following: ceftriaxone −280/+255 nm, cephaloridine +273/−260 nm, cephalexin +277/−265 nm, ampicillin −238/+233 nm, carbenicillin −237/+232 nm, where − and + represent minima and maxima, respectively.

Standard solutions

For each drug the peak–trough amplitude was measured as a function of the concentration in the sample. Each drug concentration was tested in a quartz cuvette (1 cm pathlength) using a total volume of 2 ml of solution and every measurement was repeated three times under the same conditions.

Sample solutions

Forty microlitres of a suspension of drug-loaded liposomes were diluted to 2 ml with buffer and analysed as reported above. The UV second derivative spectra of cephalosporins and the third derivative spectra of penicillins were found to be suitable (Figs 1 and 2). The measured amplitudes are shown in Table 1.

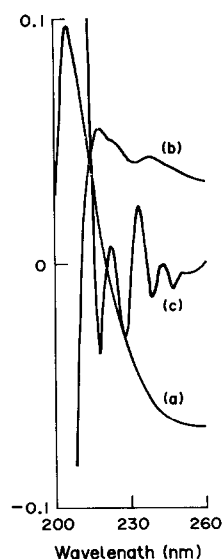

Figure 1
Zero-order (a), second-order (b) and third-order (c) derivative spectra of ampicillin.

Table 1
Regression analyses of antibiotic concentrations versus peak–trough amplitudes

β-Lactams (concentration range)	Derivative order	Regression curve (a)	r	Regression curve (b)	r
Ampicillin (25–500)	$^{3}D_{+233/-238}$	$y = 0.0259x + 0.05$	0.999	$y = 0.0275x - 0.22$	0.992
Carbenicillin (25–200)	$^{3}D_{+232/-237}$	$y = 0.0722x - 0.74$	0.998	$y = 0.0651x - 0.39$	0.997
Ceftriaxone (10–160)	$^{2}D_{+255/-280}$	$y = 0.0852x + 0.53$	0.992	$y = 0.0876x + 0.74$	0.991
Cephaloridine (20–240)	$^{2}D_{+273/-260}$	$y = 0.0536x - 0.65$	0.988	$y = 0.0499x - 0.53$	0.991
Cephalexin (10–300)	$^{2}D_{+277/-265}$	$y = 0.0404x + 0.01$	0.999	$y = 0.0354x + 0.06$	0.999

Where y = peak–trough amplitude between a characteristic maximum and minimum, measured in cm; x = concentration of β-lactam in μM; r = correlation coefficient.

The samples were measured in buffer (a) or after addition of empty liposomes (b) in both cuvettes. For each drug, six different concentrations were analysed and repeated in triplicate (see Materials and Methods). The relative standard deviation ranged from 2 to 4%.

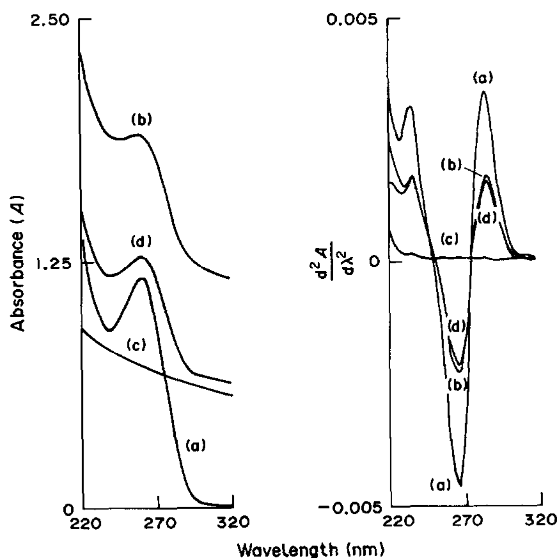

Figure 2
Zero-order (left) and second-order derivative (right) spectra of cephalexin solutions. (a) 2.0×10^{-4} M cephalexin in buffer solution; (b) cephalexin-loaded liposomes versus buffer solution in the reference cuvette; (c) empty liposome solution; (d) cephalexin-loaded liposomes versus empty liposomes solution in the reference cuvette. Experiments were carried out in 0.1 M potassium phosphate buffer (pH 7.4). The DPPC–CHO molar ratio used in the liposome preparations was 2.3:1.

Rupture of the vesicles with detergent

Two hundred microlitres of drug loaded liposomes were treated with an equal volume of sodium cholate (100 mg ml^{-1}) and vortexed for 3 min. The suspension was placed for 3 h in a bath thermostated at 37°C and then centrifuged for 30 min at 12,000*g*. The clear solution was recovered and 80 μl of this was tested as described above for the sample solutions. Spectral analysis of antibiotic solutions treated with sodium cholate under the same experimental conditions did not show any decomposition.

Treatment of vesicles with β-lactamase

Broad spectrum β-lactamase from *C. diversus* was obtained as previously described [22]. Incubation of loaded liposomes with this enzyme was performed as follows: 0.5 ml of a suspension of loaded liposomes was incubated for 3 h at 37°C with 0.1 mg of β-lactamase. The suspension was washed three times with 10 ml of buffer to remove the enzyme and, finally, the liposome pellet was redissolved in 0.5 ml of buffer and tested as described above. Controls were performed to check that no spontaneous hydrolysis occurred during incubation in the absence of β-lactamase.

Results and Discussion

The spectral behaviour of β-lactam antibiotics carried by liposomes was investigated under different experimental conditions. Figure 2 shows the absorption spectra of a model antibiotic, cephalexin, in the 220–320 nm range, free in solution or entrapped in liposomes. The zero-order spectra (left) show the characteristic peak of the drug in

buffer [curve (a)], which although detectable, is not measurable when the antibiotic is entrapped in liposomes because of the interference due to the liposomes [curves (b) and (d)].

In contrast, the same samples analysed in the second-order derivative mode (Fig. 2 right) indicate that liposomes do not interfere with the analysis and the peak–trough amplitudes can be used as a measure of the entrapped drug. Similar results were found with all other antibiotics tested. Table 1 summarizes the derivative modes chosen for all the antibiotics tested and the appropriate wavelength range for each; the regression analyses for series of standard solutions are also reported.

Interestingly, the amount of antibiotic measured after rupture by means of the detergent sodium cholate is in good agreement with that measured in the whole liposomes, demonstrating that the concentration of drug determined by the derivative method corresponds to that of the antibiotic entrapped in the liposomes. Figure 3 shows the relationship between the peak height of entrapped ampicillin and drug concentrations in the range 25–500 μM. At higher antibiotic concentrations, a saturative phenomenon is observed, which is common to all the β-lactams tested. Data from these curves indicated that the encapsulation efficiency of all drugs tested was related to the solute concentrations used in the liposome preparation; however, the efficiency of encapsulation varied from drug to drug. Different ratios of entrapped/free antibiotic have been observed as a general phenomenon in many other systems [23].

Release of penicillins but not cephalosporins from loaded liposomes occurred, the former in the order of 20% of the total amount of the encapsulate after 24 h at room temperature. This evidence, which confirms that penicillins, but not cephalosporins, can permeate the liposomes, is in good agreement with the results obtained by Hiruma *et al.* [24]. The influence of the CHO–DPPC ratio in the lipid mixture on the concentration of carried antibiotic was also studied. The liposomes without CHO were found to possess the highest concentration of drug (Table 2). Preliminary results were obtained to ascertain whether some antibiotics might be bound to the surface of the liposomes.

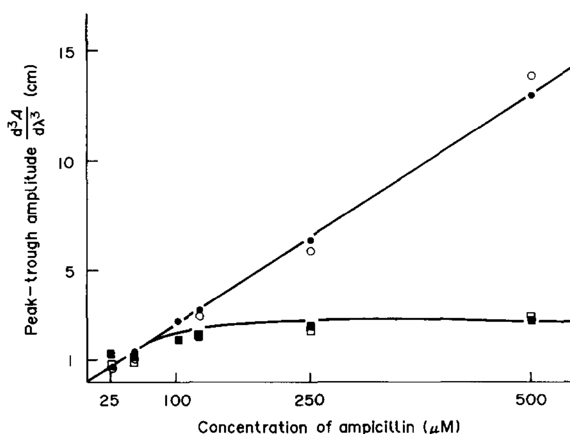

Figure 3
Relationship between the third derivative amplitude and concentration of ampicillin. ●, standard solutions; ○, standard solutions with empty liposomes; ■, antibiotic-loaded liposome suspensions; □, loaded liposomes after disruption by detergent. Each point corresponds to the average of three measurements.

Table 2
Concentration of entrapped cephalexin as a function of cholesterol concentration in the lipid mixture

DPPC–CHO (molar ratio)	Antibiotic concentration (μM)							
	25*	(—)	50*	(—)	75*	(—)	150*	(—)
1:1	3.1	(12.4)	4.4	(8.8)	11	(14.6)	15	(10.0)
2.3:1	6.8	(24.8)	9.0	(18.0)	17	(22.6)	35	(23.3)
5.6:1	14	(56.0)	19	(38.0)	36	(48.0)	80	(53.3)
10:0	17	(68.0)	31	(62.0)	51	(68.0)	90	(60.0)

*Drug concentration used in the liposome preparation.
Values in parentheses refer to the percentage of drug vehiculated.

Liposomes treated with external β-lactamase were found to possess an antibiotic concentration about 20–30% lower in the case of 2.3:1 DPPC–CHO molar ratio, compared to that of untreated liposomes.

Acknowledgements — Authors thank Professor Roberto Strom (University of Rome) for his interest in the work and helpful discussion and Dr Mauro Maccarrone (University of L'Aquila) for the critical revision of the manuscript. This work was partially supported by MPI 60% grant.

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[*Received for review 9 February 1989; revised manuscript received 12 May 1989*]