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Note

Properties of acyclovir-containing liposomes for potential ocular delivery

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Abstract

Loading efficiency and release characteristics of acyclovir-containing liposomes were investigated in this study. It was found that positively charged liposomes gave the highest loading efficiency. The neutral liposomes showed a loading efficiency in between those of the positively charged and negatively charged liposomes. Liposomes prepared with an acyclovir concentration of 5 mg/ml demonstrated a loading efficiency greater than that with an acyclovir concentration of 1 mg/ml. Liposomes prepared by the method of drug–lipid film hydration with medium presented a higher loading efficiency than that prepared by lipid film hydration with drug solution. For all liposomes, the release rate was faster at pH 7.4 than at pH 8.0. For positively charged liposomes, the release rate was faster at higher molar ratios of stearylamine. However, for negatively charged liposomes, the release rate with a molar ratio of dicetylphosphate at 0.15 was greater than at 0.3. Decrease of mobility was found for positively charged liposomes after loading with acyclovir, whereas, no significant change in mobility was observed for negatively charged and neutral liposomes.

Keywords: Acyclovir; Liposome; Loading efficiency; Release; Electrophoretic mobility

1. Introduction

Acyclovir (ACV), an antiviral drug, is common in clinical use for the treatment of herpes virus. Due to its limited solubility in both water and lipid, ACV has been developed as both water soluble prodrugs (Bundgaard et al., 1991; Giammona et al., 1995) and lipophilic prodrugs (Tong et al., 1991). It has also been formulated in liposomal dosage forms to improve its delivery characteristics (Norley et al., 1986; Ho et al., 1987; Tong et al., 1991). ACV was encapsulated in target-sensitive immunoliposomes composed of

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antibody (palmitoyl conjugated IgG against herpes simplex virus) and egg phosphatidylcholine, or transphosphatidylated phosphatidylethanolamine. An in vitro virus inhibitory effect with less cell cytotoxicity was evident (Norley et al., 1986; Ho et al., 1987). An enhancement of antiviral activity of a lipophilic prodrug of ACV in egg PC was found in cell cultures (Tong et al., 1991). Liposomes can thus be used as an efficient carrier for the delivery of ACV.

Liposomes can be formulated from a variety of lipid and lipid mixtures with different compositions and can be modified in particle size, structure and surface charge to obtain desirable physicochemical properties to suit particular needs. Since ACV cannot be given as eyedrops, attempts to use liposomes to entrap ACV as an ophthalmic dosage form have been a great interest in drug delivery research. In this study, we therefore focused on the development of a formulation for ACV-containing liposomes by modifying the liposomal surface charge, added charge inducing agent concentration, added ACV concentration, pH of the medium, and methods of preparation liposomes to investigate the loading efficiency and release characteristics. These results may provide some practical information for the development of a liposome ocular delivery system to promote ACV absorption in future studies.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC; Type XI-E: from fresh egg yolk) was obtained from Sigma, USA. Stearylamine (S) and dicetylphosphate (DP) used as cationic and anionic charge inducing agents, respectively, were obtained from PL Chemicals, Sweden. Acyclovir (ACV) and cholesterol (C) were purchased from Sigma, USA. General chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of ACV-containing liposomes

Two methods were used to prepare the ACV-containing liposomes. The first was a method of drug–lipid film hydration. A methanol and chloroform (1/1, w/w) mixture was added to dissolve the ACV and liposome ingredients of PC, C and charge inducing agents. PC and C were added in a molar ratio of 1.6 and 1, respectively. Charge inducing agents were used in various molar ratios from 0.15 to 1.6. The resultant solution was dried to form a film on a round bottomed flask under reduced pressure at 37°C in a rotary evaporator. Phosphate buffered saline was added to the film, and vigorous vortexing was applied for 5 min. After hydration for 24 h at 4°C, the dispersion was sonicated for 1 min in a cup horn sonicator (Heat Systems–Ultrasonics, USA) at the temperature of 25°C under an atmosphere of nitrogen. The second method used to prepare the liposomes was a method of lipid film hydration with drug solution, as described previously (Law et al., 1991). A chloroform mixture of PC, C and charge inducing agents at the desired molar ratio was dried to a thin film on the round bottomed flask under reduced pressure at 37°C in a rotary evaporator. The required amount of ACV was dissolved in phosphate buffered saline and added to the film, and vigorous vortexing was applied for 5 min. After hydration for 24 h at 4°C, the dispersion was sonicated for 1 min in a cup horn sonicator at the temperature of 25°C under an atmosphere of nitrogen to form liposomes. The liposome dispersions were immediately cooled to 4°C.

The ACV-containing liposomes were separated from free (unentrapped) ACV by ultracentrifugation at 2.8×10^5 g (Beckman TLA-100, USA) for 20 min at 4°C, and washed with buffer three times.

2.2.2. Determination of loading efficiency

The concentration of entrapped ACV was determined by spectrophotometry after lysis of the liposomes with absolute alcohol. One volume of liposomes was mixed well with three volumes of absolute alcohol to dissolve the liposomes and ACV to obtain a clear solution. The UV absorbance of the sample solution was measured at

254 nm. For each determination, triplicate runs were made. The entrapped ACV concentration was expressed as loading efficiency in μmol ACV/μmol lipid.

2.2.3. Determination of phospholipid concentration

Colorimetric determination of phospholipids by means of complexation with ammonium ferrothiocyanate was used (Stewart, 1980). Liposomes were extracted and dissolved in chloroform. Ammonium ferrothiocyanate reagent was pippetted into the chloroform solution and the contents were vortexed vigorously for 20 s. The resultant mixture was centrifuged for 5 min at 1000 rpm to separate the chloroform layer. The brown colored chloroform layer was removed and measured at 485 nm for phospholipid concentration. For each determination, triplicate runs were made.

2.2.4. Particle size analysis of liposomes

The particle size of the liposomes was analyzed by a Laser Particle Analyzer system (LPA-3000, Photal, Otsuka, Japan).

2.2.5. Electrophoretic mobility measurement of liposomes

Electrophoretic mobility measurement was used to determine the change of the surface charge characteristics on liposomes affected by ACV during the loading process. The experiment was carried out using a Rank MK II Microelectrophoresis Apparatus (Rank, UK). The liposomes were prepared in 10^{-3} M NaCl solution to provide a medium of suitable conductance and to maintain a constant ionic strength. The pH of the dispersions was adjusted to the required value by HCl and/or NaOH solution. A flat cell assembly and platinum electrodes were used. Ten particles were timed in both directions of the electric current to minimize the polarization of electrodes.

2.2.6. Release study

ACV-containing liposomes were suspended in buffers and incubated at 37°C in several sealed test tubes. At various time intervals, the test tubes were removed, and the liposomes were pelleted by ultracentrifugation at 2.8×10^5 g for 20 min at 4°C. After washing with buffer three times, the supernatants and rewashed liposomes were retained for measurement of ACV concentration. For each determination, three release samples were tested.

3. Results and discussion

The loading efficiency of ACV in liposomes against various molar ratios of charge inducing agents with added ACV concentration of 5 mg/ml at pH 7.4 and 8.0 is shown in Fig. 1. At pH 8.0, positively charged liposomes composed of PC, C and S demonstrated an optimal loading efficiency of 0.379 μmol ACV/μmol lipid at the molar ratio of 0.3 of S. Negatively charged liposomes consisting of PC, C and DP showed a loading efficiency with no significant change over the molar ratios of DP studied. The loading efficiency was about 0.12 μmol ACV/μmol lipid. Neutral liposomes comprising PC and C showed a loading efficiency of 0.16 μmol ACV/μmol lipid. The loading efficiency of the neutral liposomes is in between those

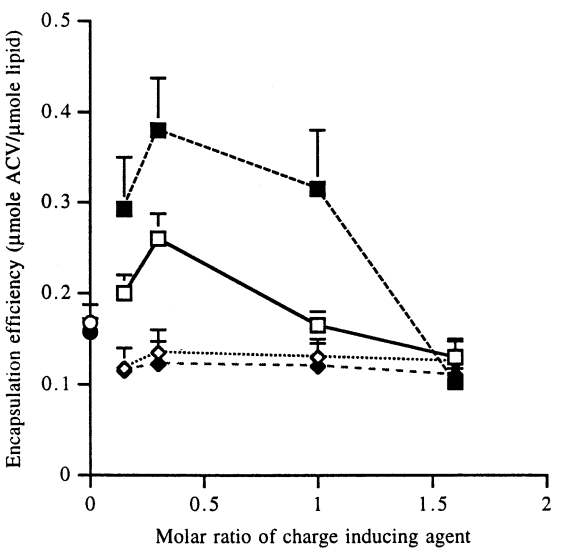

Fig. 1. Loading efficiency of ACV in liposomes with added ACV concentration of 5 mg/ml at pH 7.4 (open symbols) and 8.0 (filled symbols). Squares, positively charged liposomes (PC/C/S); diamonds, negatively charged liposomes (PC/C/DP); circles, neutral liposomes (PC/C). The molar ratio of PC/C is 1.6/1.

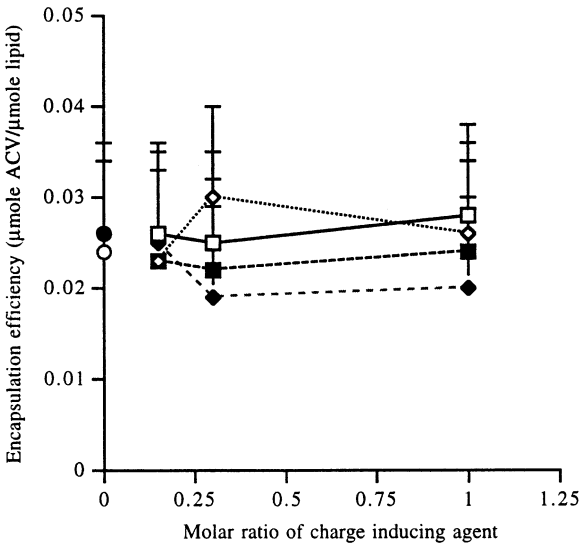

Fig. 2. Loading efficiency of ACV in liposomes with added ACV concentration of 1 mg/ml at pH 7.4 (open symbols) and 8.0 (filled symbols). Squares, positively charged liposomes (PC/C/S); diamonds, negatively charged liposomes (PC/C/DP); circles, neutral liposomes (PC/C). The molar ratio of PC/C is 1.6/1.

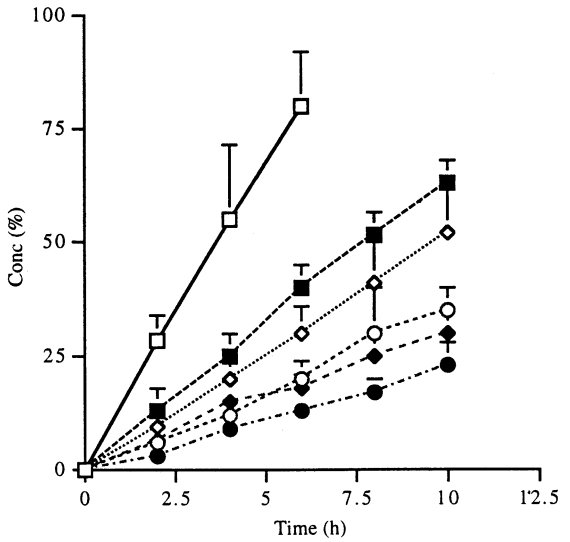

Fig. 3. Release characteristics of ACV from positively charged liposomes (PC/C/S) at pH 7.4 (open symbols) and 8.0 (filled symbols). 1.0 (squares); 0.3 (diamonds); 0.15 (circles) molar ratio of S. The molar ratio of PC/C is 1.6/1.

of positively charged liposomes and negatively charged liposomes. It is clear that the positively charged liposomes give a highest loading efficiency with added ACV concentration of 5 mg/ml.

For negatively charged and neutral liposomes at pH 7.4, similar loading efficiency was obtained as at pH 8.0. However, for positively charged liposomes, the loading efficiency is lower at pH 7.4 when compared with the loading efficiency at pH 8.0. The pK_a values for ACV are 2.27 and 9.25 (Dollery, 1991). Therefore, more ACV is ionized at pH 8.0 than at pH 7.4. The deprotonated ACV may be more active to interact with the positively charged loading compartment of the liposomes by electrostatic interaction at higher pH. It is not surprising that the loading efficiency of the positively charged liposomes is higher at pH 8.0 than at pH 7.4. The loading efficiency of the neutral liposomes was not affected by the pH which may be due to no charge interaction between the ACV molecules and the liposomes. In the case of negatively charged liposomes, loading efficiency was the lowest among the positively charged and neutral liposomes. It is likely that charge repulsion may occur with the drug molecules and negatively charged liposomes to suppress the loading efficiency.

Table 1
Effect of methods for the preparation of positively charged liposomes (PC/C/S) on loading efficiency

pH	Loading efficiency (μmol Acv/μmol lipid)	
	Drug-lipid film hydration with medium	Lipid film hydration with drug solution
8.0	0.379 ± 0.076	0.037 ± 0.019
7.4	0.260 ± 0.039	0.039 ± 0.020

The molar ratio of PC/C/S is 1.6/1/0.3.

Fig. 2 shows the effect of added ACV concentration of 1 mg/ml on the loading efficiency of liposomes with various molar ratios of charge inducing agents at pH 8.0 and 7.4, demonstrating that, in spite of the charge characteristics and molar ratio of charge inducing agents, the liposomes entrapped similar concentration of ACV. That is to say, within the studied range of molar ratio of charge inducing agents for construction of liposomes, positively charged, negatively

charged and neutral liposomes have no significant effect on the loading efficiency at a hydration ACV concentration of 1 mg/ml. Also, from Fig. 2, within experimental error, pH seems to be not a significant factor affecting the loading efficiency. These results may be due to the low entrapped concentration resulted from the low added ACV concentration. The loading efficiency at an ACV concentration of 5 mg/ml is higher than that at the 1 mg/ml level.

The effect of the method of drug–lipid film hydration with medium and lipid film hydration with drug solution for the preparation of positively charged liposomes (PC/C/S, 1.6/1/0.3) on loading efficiency is shown in Table 1. For liposomes prepared by the second method, similar loading efficiency was obtained at pH 7.4 and 8.0. However, for those prepared by the first method, the loading efficiency was higher at pH 8.0. Also, the loading efficiency obtained from the first method was higher than that from the second method. It is suggested that for the first method of preparation ACV-containing liposomes, the ACV in organic solvent is present in neutral form, and it may interact with phospholipids in the organic phase before film formation (Crommelin and van Bloois, 1983). This interaction may be significant when both ACV and phospholipid are in a dry film mixture. As aqueous medium is added, interactions become more effectively hydrophobic between the drug and the phospholipid bilayers. Therefore, more drug molecules are encapsulated in the liposomes. For the second method of preparation, the ACV was dissolved in the aqueous medium in a hydrophilic state which may interact less with the hydrophobic phospholipid bilayers leading to a low loading efficiency. For liposomes prepared by the first method, the particle size was 1286 ± 868 nm; whereas, for those prepared by the second method it was 1091 ± 745 nm. This demonstrated no significant difference in particle size.

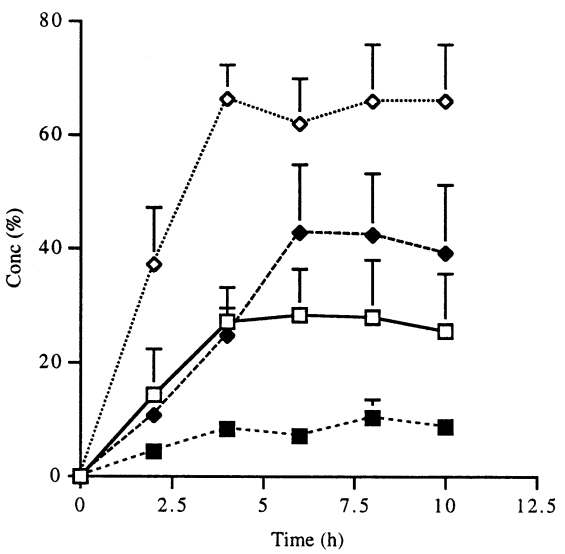

Fig. 4. Release characteristics of ACV from negatively charged liposomes (PC/C/DP) at pH 7.4 (open symbols) and 8.0 (filled symbols). 0.3 (squares); 0.15 (diamonds) molar ratio of DP. The molar ratio of PC/C is 1.6/1.

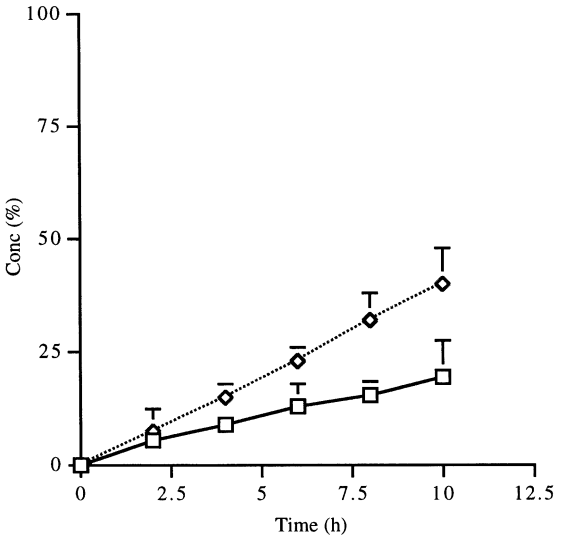

Fig. 5. Release characteristics of ACV from neutral liposomes (PC/C). pH 8.0 (squares); pH 7.4 (diamonds). The molar ratio of PC/C is 1.6/1.

Fig. 3 shows the release profiles of ACV from positively charged liposomes with various molar ratios of S at pH 7.4 and 8.0. Release increased linearly with increasing incubation time in the time range studied. The release rate was faster at higher molar ratios of S. Also, the release rate at pH 7.4 was faster than that at pH 8.0. Fig. 4 represents the ACV release characteristics for negatively charged liposomes with 0.15 and 0.3 molar

Table 2
Electrophoretic mobility of positively charged (PC/C/S), negatively charged (PC/C/DP) and neutral (PC/C) liposomes after loading with ACV

Molar ratio of charge inducing agent	Mobility (μs per v/cm)					
	PC/C/S		PC/C/DP		PC/C	
	Empty liposome	Acv-containing liposome	Empty liposome	Acv-containing liposome	Empty liposome	Acv-containing liposome
0	—	—	—	—	0	0
0.15	3.01 ± 0.10	2.80 ± 0.07	-4.82 ± 0.22	-5.05 ± 0.13	—	—
0.3	3.56 ± 0.11	3.02 ± 0.24	-6.42 ± 0.26	-6.06 ± 0.10	—	—
1	4.54 ± 0.15	3.66 ± 0.18	—	—	—	—

The molar ratio of PC/C is 1.6/1.

ratio of DP at pH 7.4 and 8.0. The increase of release concentration was shown in the initial hours, and then a plateau was found with increasing release time which resulted in a two-phase release characteristics for ACV from the liposomes. The release rate at 0.15 molar ratio of DP was greater than that at 0.3. The ACV release profiles from neutral liposomes at pH 7.4 and 8.0 are shown in Fig. 5. A linear release characteristic was obtained in the time range studied. The rate of drug release at pH 7.4 was faster than that at pH 8.0. As mentioned above, the ACV is ionized more at pH 8.0 than at pH 7.4. The ionized drugs may partition with difficulty into the lipid bilayers resulted in a slow release rate (Tsukada et al., 1984; Law et al., 1994). The effect of increase of molar concentration of charge inducing agents on the release of ACV demonstrated an increase of rate for positively charged liposomes but a decrease for negatively charged liposomes. This may be attributed to the charge interaction between the deprotonated ACV molecules and the positively, or negatively charged liposomes leading to an increase, or decrease of ACV partition into the lipid bilayers, respectively (Tsukada et al., 1984; Law et al., 1994).

Table 2 shows the electrophoretic mobility of positively charged, negatively charged and neutral liposomes after loading with ACV (results obtained at pH 7.4 not shown). It is clear that negatively charged and neutral liposomes after loading with ACV showed the same mobility as that of the empty liposomes, within experimental error. ACV exerted no effect on the surface of the negatively charged and neutral liposomes. Whereas, ACV-containing liposomes with positive charge inducing agent demonstrated an decrease of mobility when compared with the blank liposomes. The decrease of mobility indicates a suppression of surface charge which may be due to the interaction of the ionized ACV with the positive surface charge groupings of the liposomes during the loading process.

Acknowledgements

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