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SYNTHESIS AND STABILITY OF A COVALENTLY LINKED LIPOSOME-PHENOL ESTER MODEL PRODRUG

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ABSTRACT

This study examines the feasability of covalently bonding a drug to a liposomal wall component to yield a potential liposome prodrug. The model compound chosen, p-nitrophenol, was esterified with the stearic acid present in liposomes employing

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the coupling agents 1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide (EDCI) and dicyclohexylcarbodiimide (DCC). Reactions conducted with EDCI in distilled water or with DCC in either distilled water or phosphate buffer yielded >80% p-nitrophenyl stearate. When EDCI was used in the presence of phosphate buffer, the ester yield was reduced to ~40%. The time for ten percent degradation (T_{90}) of the liposome ester was evaluated at 37°C in the pH range 1-11. A ~3 to 17 fold rate enhancement was observed when esterase was added to the buffered solutions.

INTRODUCTION

Liposomes have attracted interest as potential vehicles for drug delivery (1-4). They have been reported to protect drugs from degradation in vivo (5,6), increase drug circulation time in the blood (7), and deliver drugs to specific sites in the body (8-11).

However, liposome drug delivery systems tend to concentrate in the reticuloendothelial system thus impeding delivery to other sites (12). They may also suffer increased permeability with enhanced release of their encapsulated contents on contact with plasma or serum (12,13) owing to interactions of the phosphatidylcholine with high density lipoproteins (14,15).

Increasing the cholesterol in the lipid bilayer has been partially successful in overcoming this problem (16).

The present study investigates the feasability of covalently bonding drugs to functional groups on the liposomal surface. Drugs anchored in this manner might then remain with the liposomes during plasma circulation. The bonding of p-nitrophenol (chosen as a model compound) to stearic acid present in liposomes was investigated under various conditions in the presence of coupling agents. Hydrolysis of the liposome-nitrophenol ester was evaluated in buffered solutions to determine the influence of pH on storage stability and in the presence of esterase to establish enzymatic rate enhancement.

METHODS

Synthesis of liposome-nitrophenol ester. Liposomes were prepared from L-α-phosphatidylcholine ($13\text{-}14 \times 10^{-3}$M) and stearic acid ($6.5\text{-}7.0 \times 10^{-3}$M) in the molar ratio of 2:1 using the surface film-sonication method (17). Nitrogen-saturated aqueous solvents and flushing of the headspace with nitrogen gas were employed throughout the procedure. The model compound, p-nitrophenol, was covalently linked to the stearic acid present in the liposomes to form the ester p-nitrophenyl stearate using the coupling agents dicyclohexylcarbodiimide (DCC) or

1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDCI). Yields of p-nitrophenyl stearate were determined as a function of time at room temperature and ~5°C in the presence and absence of 0.1 M phosphate buffer (pH = 7.7).

Qualitative tests for formation of the ester (R_f = 0.78) were performed with 2.5x10 cm TLC plates (Silica Gel Uniplates, 250 uM; Analtech Inc.) developed with chloroform acidified with glacial acetic acid and visualized using iodine vapors. Sample preparation involved extraction of the liposomal suspension with chloroform and phase separation followed by drying of the organic phase with sodium sulfate.

The amount of p-nitrophenol covalently bonded to stearic acid in liposomes was estimated by analyzing for the total p-nitrophenyl stearate in the suspension using UV spectroscopy. Prior to analysis, a 0.05-0.25 mL aliquot of the liposomal suspension was dissolved in 5.0 mL of 0.5 M formic acid in methanol and the UV spectrum recorded. Formation of the ester was indicated by the loss of the original peak at 313 nM and the appearance of a new peak at 269 nM. The total concentration of the ester and the unreacted phenol was obtained using the following analytical techniques.

The liposome-nitrophenol ester used for stability testing was synthesized in distilled water at room temperature using EDCI as

the coupling agent based upon preliminary screening studies. Liposomes were prepared in 9 mL of nitrogen-saturated distilled water. A 0.2 mL aliquot of 9×10^{-3} M. p-nitrophenol methanolic solution was added to the suspension followed by 20 mg of EDCI (11.6×10^{-3} M) and the mixture was agitated overnight. The suspension was then ultracentrifuged (Beckman L5-50B Ultracentrifuge) at 41,000×g (≃105,000 rpm), 4°C for 30 minutes to form a pellet. The supernatant (≃7 mL) was removed and replaced with an equal volume of fresh distilled water and the procedure repeated. Each supernatant was analyzed for ester and phenol. The liposomal pellet was then dispersed in the remaining aqueous solvent. This suspension was subjected to dialysis for 20-24 hours against 200 mL of distilled water with 3 changes of external dialysis fluid at equal intervals. The dialysis bag was replaced with a new one after approximately 12 hours. Suspensions were assayed for ester concentration after each stage of the preparation: (1) overnight agitation, (2) ultracentrifugation and (3) dialysis. Ten batches of liposome p-nitrophenol ester prepared according to the above procedures were combined and used for further studies.

Analytical. The assay for total p-nitrophenyl stearate and total p-nitrophenol in liposomal suspensions were performed spectrophotometrically (Beckman DU-7 spectrophotometer) as

follows. Molar absorptivities in methanol containing 0.5 M formic acid at 269 and 313 nm were 10.1×10^3 and 1.36×10^3 for the ester and 3.00×10^3 and 10.7×10^3 for the phenol. Concentrations of the ester (C_1) and phenol (C_2) in the final dilutions were calculated from

$$10^5 C_1 = 10.3\ A_{269} - 2.89\ A_{313} \quad (1)$$

$$10^5 C_2 = 9.98\ A_{313} - 1.31\ A_{269} \quad (2)$$

derived from simultaneous equations for total absorbance, A, at 269 and 313 nM.

For stability studies of the liposomal-nitrophenol ester in buffers, a 0.3 mL aliquot of the liposomal suspension was added to 4.0 mL of 0.5 M formic acid in methanol (to dissolve the liposomes and quench the hydrolysis of the ester) and the absorbance was measured against a blank prepared in a similar manner. The susceptibility of the liposome ester to esterase-catalyzed hydrolysis was examined using identical procedures except that the formic acid solutions were centrifuged at 3200 rpm for 10 min prior to analysis.

Stability in buffers. The time for 10% degradation of the liposomal ester (T_{90}) was estimated in acetate, phosphate, tris, carbonate buffers and HCl at $\mu = 0.4$ (adjusted with NaCl) in the pH range 1-11 at 37°C. Liposome ester suspension (0.4 mL) was

dispersed in 3.6 mL of buffer at 37°C in a temperature controlled shaker bath. Reactions were analyzed for liposome ester and p-nitrophenol as a function of time.

Susceptibility to esterase. Enzyme-catalyzed hydrolysis of the liposome ester was studied in the pH range 7.5-9.5 at 37°C. Pig liver esterase (carboxylic ester hydrolase, EC 3.1.1.1., Sigma) was standardized using p-nitrophenyl acetate as the substrate. The enzyme was diluted 100 fold and 0.1 mL of this dilution added to a 1.6 mM solution of p-nitrophenyl acetate in phosphate buffer at pH 7.23, 25°C. The p-nitrophenol released was measured by UV analysis (17, 18). Initial rates of the concentration-time profiles were used to determine relative enzyme activity. One unit was defined as that amount which was found to hydrolyze 10 mL of a 1.6 mM p-nitrophenyl acetate solution at the rate of 1 μmole/min at 25°C, pH 7.23.

To initiate the enzyme catalyzed reaction, 0.4 mL of ester suspension was diluted with 3.6 mL of buffer to which was added 25 units of the enzyme (~0.11 mL) using a microburette. The reaction was analyzed as a function of time for total ester and phenol.

RESULTS AND DISCUSSION

Liposomal ester preparation. The yield of liposomal p-nitrophenyl stearate formed in the presence of carbodiimide coupling agents was expressed as

$$\% \text{ p-nitrophenol reacted} = 100 \times C_1/(C_1+C_2) \quad (3)$$

where C_1 is the measured concentrations of ester and and C_2 the unreacted phenol. The sum of the ester and the excess p-nitrophenol concentration was equal to the initial concentration of p-nitrophenol. The percent reacted is shown as a function of time for each of six conditions in Fig. 1. Reactions in distilled water using EDCI at room temperature reacted ~80% of the p-nitrophenol. The identical reaction conducted under refrigeration, previously reported to give significant yields of covalently bonded product (20), reduced values to ~60% during the same time. No significant difference in the maximum yields (~80%) were obtained between DCC and EDCI reactions.

Only 30% of the p-nitrophenol was found to react in phosphate buffer at room temperature using EDCI. This represents a 2.7-fold decrease relative to distilled water. A similar reduction was observed in phosphate buffer under refrigerated conditions. The pH 7.7 buffer, having a higher pH than distilled water at ~5.1, might reduce yields by hydroxyl ions competing with stearic acid in forming the reactive intermediate, with p-nitrophenol in the subsequent displacement step, or by increasing the hydrolysis of the ester after it is formed.

The maximum percentage of p-nitrophenol reacted (~80%) did not change when phosphate buffer was used in the DCC

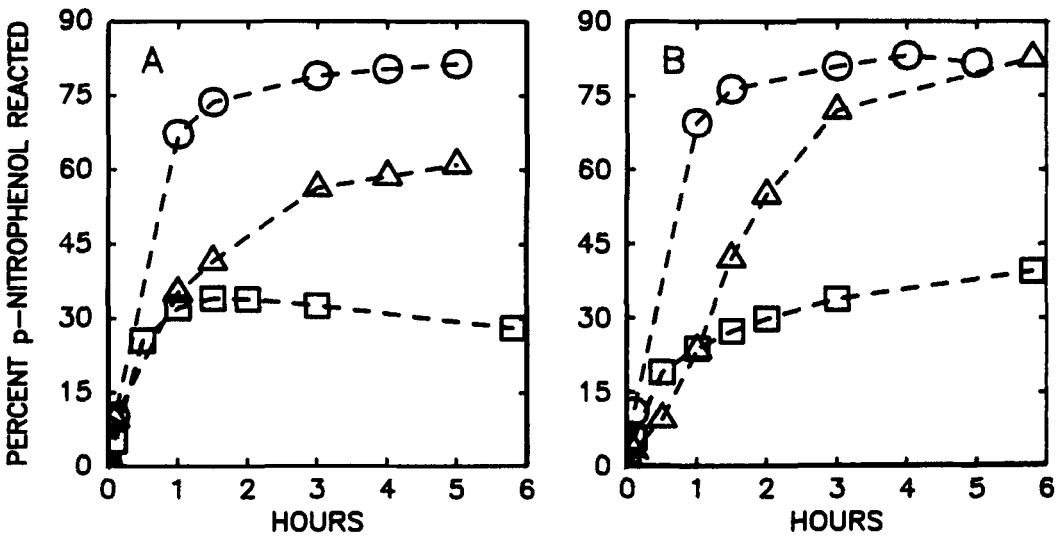

FIGURE 1

The percentage p-nitrophenol reacted as a function of time under the following conditions:

(1) EDCI in water at room temperature

(2) at ~5°C.

(3) DCC in water at room temperature

(4) EDCI in phosphate buffer, ph = 7.7 at room temperature

(5) at ~5°C.

(6) DCC in phosphate buffer at room temperature

In A, conditions are: 1(o), 2(Δ) and 4(□). In B, conditions are: 3(o), 6(Δ) and 5(□).

reactions. This was the only contrasting behavior noted between EDCI and DCC. The higher lipid solubility of DCC may provide an increase in its effective reactive concentration within the liposomes thus reducing the ability of hydroxyl ions to compete with carbodiimide in the lipid phase.

Although both EDCI and DCC reactions in distilled water at room temperature were successful, EDCI was selected because the excess unreacted EDCI and the urea by-product could be easily separated from the liposomes using dialysis and/or centrifugation owing to their high aqueous solubility.

Liposomal ester stability. Hydrolysis of the liposomal ester was studied as a function of time in various buffers in the pH range 1-11 at 37°C. The concentration-time plots for ester were fit using the regression equation from SAS (21).

$$C_1 = W + Xt + Yt^2 + Zt^3 \qquad (4)$$

where C_1 is the concentration of ester, W,X,Y,Z are the adjustable parameters, and t is the time. The time for 10% hydrolysis of the liposome-ester (T_{90}) was calculated from the final equation for the curve of best fit using computer reiteration to solve for $C_1 = 0.9\ C_o$. A similar treatment based on phenol production gave the same results.

Figure 2 is a semi-logarithmic plot of T_{90} values as a function of pH. A direct comparison between the T_{90} values for p-nitrophenyl stearate in liposomes to those in a simple aqueous solution was not feasible owing to the low aqueous solubility of the ester precluding the determination of T_{90} values. However,

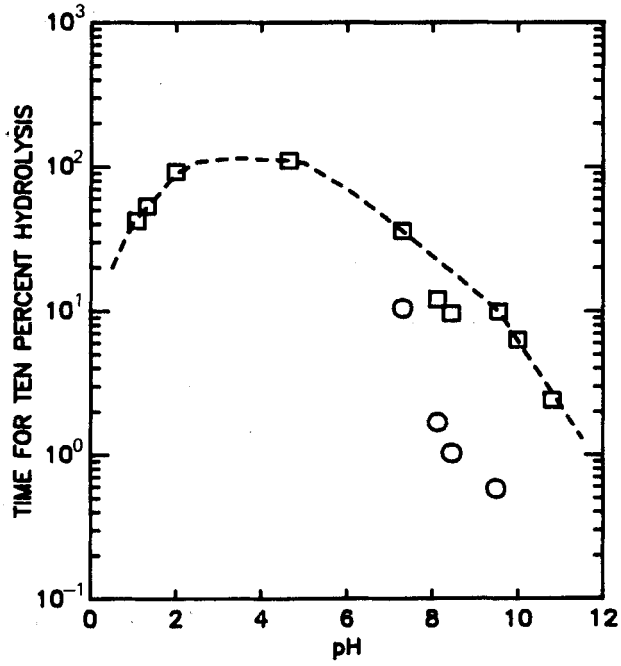

FIGURE 2

Semilogarithmic plot of the time for ten percent hydrolysis (in hours) of 1.1 x 10^{-3} M liposomal p-nitrophenyl stearate as a function of pH in buffered aqueous solutions μ = 0.4 with (o) and without (□) esterase at 37°C.

it is expected to be similar to that of the more soluble long chain ester, p-nitrophenyl octanoate. The T_{90} value for the hydrolysis of p-nitrophenyl octanoate in solution at 37°C, pH 9.5, calculated from a previous report (22), is 1.5 min. If this value is approximately the same for p-nitrophenyl stearate, then ester associated with liposomes (T_{90} = 9.87 hours) effects a ~400 fold increase in stability relative to that in an aqueous solution.

The T_{90} values obtained for the liposomal ester at pH 8.12 and 8.45 in Tris buffers appear to be lower than the dashed curve showing the trend of the other points in Figure 2. Enhanced loss of p-nitrophenyl acetate in the presence of liposomes dispersed in Tris buffers has previously been reported (23). The buffer, tris(hydroxy methyl) amino methane, may act as a nucleophile (24) resulting in aminolysis of the liposome-ester.

Since several penicillin ester prodrugs are known to convert instantly in the presence of esterase (25-27), the susceptibility to esterase-catalyzed hydrolysis was investigated in the pH range 7.3-9.5 at 37°C. The rate of formation of p-nitrophenol increased as the pH was increased to pH 9.5 where the T_{90} value was 16 fold greater in the absence of esterase (Figure 2). The T_{90} value of 10.5 hours at pH 7.30 suggests that the liposome prodrug is sufficiently stable to circulate intact in vivo. If the ester circulates intact, the liposome prodrug would be available for drug release subsequent to phagocytic or adsorptive cellular uptake of the liposomes per se.

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