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Liposomal Formulations of Serratiopeptidase: In Vitro Studies Using PAMPA and Caco-2 Models

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Abstract: The feasibility of using liposomes as a potential oral delivery system for the systemic delivery of other peptides and protein-based pharmaceuticals has been studied. Serratiopeptidase, a proteolytic enzyme, was used as a model drug. Liposomes were prepared by a thin film hydration method using various lipids, namely, soya lecithin, DMPC and DMPE. It was further investigated whether the liposomal formulations of serratiopeptidase altered the permeability/absorption of the drug using PAMPA, a non-cell-based assay, and Caco-2 assay, a cell monolayer system, mimicking in vivo GI epithelium cells. The entrapment efficiency of the formulations was found to be 62%, 84% and 86% for the liposomes of soya lecithin, DMPC and DMPE respectively. The effectiveness of the liposomal formulations against the pure drug in terms of permeability/absorption was compared. The effective permeability (log P_e) values from PAMPA study varied from −7.47 to −6.5 cm/s whereas for the serratiopeptidase it was −7.72 cm/s. The apparent permeability values calculated from Caco-2 assay varied from 1.25×10^{-6} to 1.61×10^{-6} cm/s whereas for the serratiopeptidase it was 1.25×10^{-6} cm/s. The flux was found to be 3.88–4.96 μg/cm^2/h for the formulations when compared to 3.208 μg/cm^2/h for serratiopeptidase. The results obtained indicated that in comparison with the pure drug, incorporation of drug into liposomes improved the permeability. Thus it could be concluded that the liposomal formulations would improve the oral absorption of serratiopeptidase.

Keywords: Liposome; soya lecithin; DMPC; DMPE; serratiopeptidase; PAMPA; Caco-2

Introduction

Protein and peptide formulation is rapidly becoming an essential part of drug development strategy. Unfortunately, the majority of these require daily multiple injections. Establishing an oral delivery system for peptides and protein drugs is of great importance because parenteral administration results in poor patient compliance during chronic treatment, resulting in limited clinical utility. The primary problems encountered with the oral delivery of protein pharmaceuticals include the poor intrinsic permeability owing to their hydrophilic nature and large molecular size, presystemic enzymatic metabolism by intestinal proteases and peptidases, chemical instability including tendencies to aggregate, and/or nonspecific binding to a variety of physical and biological surfaces.[1,2]

The feasibility of using liposomes as a potential oral delivery system for the systemic delivery of other peptides and protein-based pharmaceuticals has been studied. Serratiopeptidase is used as a model drug. Serratiopeptidase, also known as serrapeptase, is a proteolytic enzyme isolated from the nonpathogenic enterobacteria *Serratia* spp. When consumed in unprotected tablets or capsules, the enzyme is

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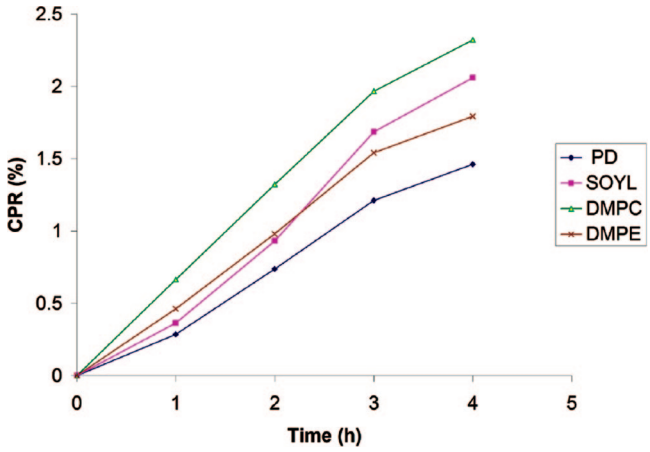

Figure 1. Release rate of drug from liposomal formulations across a Caco-2 monolayer.

destroyed by acid in the stomach.[3,4] Serratiopeptidase is an endopeptidase, having a molecular weight of about 60 kDa. Serratiopeptidase is well-tolerated. Due to its lack of side effects and good anti-inflammatory capabilities, serrapeptase is a logical choice to replace harmful NSAIDs.

Pharmacokinetic factors such as absorption/permeability across the GI epithelium become crucial. In vitro experiments like parallel artificial membrane assay (PAMPA) and cell monolayer systems such as Caco-2 mimic in vivo GI epithelium cells and currently enjoy widespread popularity. PAMPA is a non-cell-based assay which measures passive permeation, which is the major route for passage of compounds from the GI tract into the body and is thus designed to predict the oral absorption of drug candidates. PAMPA assay is a robust and reproducible assay. It is relatively fast (4–16 h), inexpensive, and straightforward. The majority of drugs (>80%) enter the blood stream by passive diffusion, and the permeation values obtained correlate with human drug absorption values from published methods.[5,6]

Caco-2 systems possess active transporters, efflux systems, and carrier-mediated transporters and, thus, mimic the multimechanism system of the intestine. However, the preparation of a fully functional monolayer requires up to 3 weeks and is thus labor intensive. Moreover there are variations in Caco-2 results due to different culture conditions making it difficult to compare findings from different sources.[7–9]

The main aim of the present investigation was to investigate whether the liposomal formulations of serratiopeptidase altered the permeability/absorption of the drug using PAMPA and Caco-2 models. Further, the feasibility of using liposomes as a potential oral delivery system for serratiopeptidase can be studied. The lipids considered in this study include phosphatidylcholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE).

Materials and Methods

Materials. Serratiopeptidase was obtained as a gift sample from MicroNova Pharmaceuticals, Bangalore. Soya Lecithin (for liposome preparation) was purchased from Himedia Laboratories Pvt Ltd., Mumbai, cholesterol was purchased from Loba chemie Pvt Ltd., Mumbai, and DMPC and DMPE were gift samples from Genzyme Pharmaceuticals, Switzerland. Ultrapure water was obtained by reverse osmosis through a Milli-Q system (Millipore). All other reagents and solvents were of the highest purity available. Propranolol, warfarin, carbamazepine, L-α-phosphatidylcholine (for PAMPA), *n*-dodecane, dimethyl sulfoxide, phosphate-buffered saline (PBS), and Hanks balanced salt solution (HBSS) with sodium bicarbonate (catalog no. H9269) were purchased from Sigma Chemical Co. MultiScreen-IP PAMPA assay plates and PTFE Acceptor plates were purchased from Millipore Corp. Dulbecco's MEM, nonessential amino acids, HEPES, penicillin, streptomycin, and L-glutamine, EDTA, and trypsin/EDTA were all purchased from Sigma Chemical Co. Fetal bovine serum was purchased from Invitrogen.

Preparation of Liposomal Formulations. Liposomes were prepared by the thin-layer evaporation (TLE) method. Briefly, lipids were dissolved in a chloroform/methanol mixture (2:1 v/v) in a round-bottom flask attached to a rotary evaporator flask (model: Rotoevaporator, Laborota 4010/4011 digital Hiedolph instruments). The organic solvent was evaporated at a temperature not exceeding 45 °C under vacuum so that the entire amount of organic solvent was removed and no residual solvent was left behind. The rotation was continued for 15 min after the dry residue first appeared to remove traces of the solvent. The lipid film was then stored closed at room temperature. Hydration of the lipid was carried out using phosphate-buffered saline (PBS pH 7.4)

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containing the drug to be entrapped. The flask was then rotated at room temperature and pressure for 30 min until the entire lipid had been removed from the wall of the flask and gave a homogeneous milky-white suspension free of visible particles. The suspension was allowed to stand overnight under refrigeration to complete the swelling process. Multilamellar vesicles were obtained. The multilamellar vesicles were extruded through polycarbonate filters (1 μm, 0.1 μm Nucleopore, Pleasanton, CA) mounted in the mini-extruder (LiposoFast, Avestin, Inc.) to get large unilamellar vesicles which was used in the study.[10,11]

Entrapment efficiency: The liposome suspension was ultracentrifuged at 12000 rpm for 1 h at 4 °C to separate the free drug. Further, the liposomes were washed with buffer and centrifuged at 12000 rpm for 1 h. This process was repeated twice so that the entire free drug was removed. The supernatants were combined and estimated for the free drug content against a formulation blank processed in a similar way. The pellet containing only liposomes was resuspended in PBS pH 7.4 until further processing. The liposomes free from entrapped drug were soaked overnight in 10 mL of methanol and then sonicated for 10 min. The vesicles were broken to release the drug, which was then estimated for the drug content against a formulation blank processed in a similar way. The absorbance of the drug was noted at 278 nm. The entrapment efficiency was then calculated using eq 1.[12]

$$\text{entrapment efficiency} = \frac{\text{entrapped drug} \times 100}{\text{total drug added}} \quad (1)$$

Permeation Studies Using PAMPA.[13,14] The following protocol was used to determine log P_e values. A 1% solution (w/v) of lecithin in dodecane was prepared and the mixture sonicated to ensure complete dissolution.

Five milliliters of the lecithin/dodecane mixture was then carefully pipetted into each donor plate well, avoiding pipet tip contact with the membrane. Immediately after the application of the artificial membrane, 150 μL of drug-containing donor solution (standard drugs namely propranolol, warfarin, carbamazepine, and liposomal formulations of serrapeptase dissolved in 5% DMSO, PBS) was added to each well of the donor plate. Buffer (300 μL; 5% DMSO in PBS, pH 7.4) was added to each well of the PTFE Acceptor plate. Then the drug-filled donor plate was placed into the acceptor plate, making sure the underside of the membrane was in contact with the buffer. The plate lid was replaced and incubated at room temperature for 16 h. The assembled plate was placed into a sealed container with wet paper towels to avoid evaporation. After incubation, samples from the donor and acceptor plate were analyzed after transferring to a UV-compatible 96-well plate and analyzed immediately at 230 nm to estimate for the drug content.

An equilibrium plate (compounds at the theoretical equilibrium, ie., the resulting concentration if the donor and the acceptor solutions are combined) was also created and analyzed. This equilibrium plate was used to calculate the permeability rate (log P_e) of the formulations when compared to the pure drug.

Cell Culture. A Caco-2 cell line was used for the experiment. Caco-2 cells were maintained at 37 °C under 5% CO_2 and 95% relative humidity in minimum essential medium (MEM). Cell culture medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), 0.1 mg/mL of streptomycin, 100 units of penicillin, 10 mM HEPES buffer, NEAA. The culture medium was changed every second day for approximately 5–6 days until the cells reached approximately 80–90% of confluence. After the passage operation, the cells were seeded at approximately 2.5×10^5 cells per flask. For this experiment, Caco-2 cells at passage number 67 was used.

Permeation Studies through the Caco-2 Cells.[15,16] At the end of the desired growth period, the plates were removed from the incubator and allowed to equilibrate to room temperature, approximately 1 h. The integrity of the cell monolayer was further verified by measuring the TEER (transepithelial electrical resistance), i.e., the potential difference between the two sides of the monolayer using a Millicell ERS meter (Millipore, Bedford, MA) connected to a pair of chopstick electrodes. Average TEER values for untreated cell monolayers were over 128–143 Ω.

The hydrophilic fluorescent marker Lucifer yellow (Sigma) was employed as a paracellular marker for checking the junction integrity of Caco-2 cells during the experiments. One hundred microliters of Lucifer yellow at a concentration of 100 μg/mL was added to selected wells in a plate prior to transport studies. HBSS (250 μL) was added to the basolateral compartments of a 96-well transport analysis plate. The plate components were assembled and incubated for 1 h at 37 °C. The filter plate was removed from the transport

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analysis plate and placed into a fluorescent plate reader (FluoStar, Optima BMG labtech 96). The fluorescent absorbance was measured using wavelengths of 485 nm excitation and 520 nm emission. The percent of Lucifer yellow passage across the cell monolayer into the transport analysis plate was calculated.

The drugs chosen to validate the procedure include propanolol and methotrexate. Test solutions were prepared by dissolving a fixed amount of each compound in HBSS. For the dynamic transport experiment, filters were placed in a 24-well plate and placed in an atmosphere of 95% air and 5% CO_2 at 37 °C. The media was replaced with HBSS buffer pH 7.4 and incubated for 0.5 h until equilibration. Transport studies were done from the apical side (AP) to the basolateral (BL) direction. After equilibration, the apical side was filled with compounds to be tested prepared in HBSS (pure drug, standards propanolol and methotrexate, liposomal formulations of serratiopeptidase), and the cells were incubated for 4 h. Aliqouts of 300 μL were withdrawn at an interval of 1 h for 4 h from the BL side (the receptor compartment), replaced with an equal volume of fresh HBSS solution, and the drug content determined.

Drug Analysis of Permeation Studies. Standard Curve of Serratiopeptidase by UV Method in the Microplate Format. Standard curves of pure drug serratiopeptidase were prepared in three different buffers PBS pH 7.4 (for estimation of drug content in formulations), 5% DMSO in PBS 7.4 for estimation of samples in PAMPA assay and in HBSS for detection of drug content from Caco-2 using a microplate reader (μQuant from Biotek instruments Inc.).

Calculation of the log P_e Values for PAMPA Assay.[13,14,17,18] The assay measures the ability of the compounds to diffuse from a donor to an acceptor compartment separated by a PVDF membrane pretreated with a lipid containing solvent.

Log P_e can be calculated from the equation as reported by Faller

$$\log P_e = \log\left(C \times -\ln\left[\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}}\right]\right) \qquad (2)$$

where

$$C = \frac{V_D \times V_A}{(V_D + V_A)A \times \text{time}}$$

where V_A is the volume in the acceptor well, V_D is the volume in the donor well, and A is the "effective area" of the membrane insert. Effective area is the surface area multiplied by the porosity ratio (20% for polycarbonate and 100% for PVDF), $[\text{drug}]_{\text{acceptor}}$ refers to the concentration of drug in the acceptor compartment, $[\text{drug}]_{\text{equilibrium}}$ is the concentration of drug in the equilibrium mixture, and time is the total transport time in seconds.

Table 1. Particle Size and Entrapment Efficiency (%) of the Formulations

formulation	drug/lipid ratio	size (nm)[a]	encapsulation efficiency (%)[a]
soyalecithin liposomes	1:1	78 ± 0.18	62 ± 0.15
DMPC liposomes	1:1	105 ± 0.11	84 ± 0.23
DMPE liposomes	1:1	120 ± 0.25	86 ± 0.43

[a] Average of six determinations.

Calculate the Apparent Permeability for Caco-2 Assay.[15,17,19] The assay measures the ability of the compounds to diffuse from a donor to an acceptor. The apical to basal apparent permeability coefficient was calculated according to the equation

$$\text{apparent permeability } P_{app} = \frac{V}{\text{area} \times \text{time}} \times \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{initial,donor}}} \qquad (3)$$

where V is the volume of the acceptor well in milliliters (0.6 mL), A is the area of the membrane insert (0.6 cm^2), $[\text{drug}]_{\text{acceptor}}$ is the concentration of drug in the acceptor compartment, $[\text{drug}]_{\text{initial,donor}}$ is the initial drug concentration added to the donor compartment, and time is the total transport time in seconds.

Results

Entrapment Efficiency. A linear relationship between drug concentration and absorbance was observed between 1 and 4 μg/mL at 230 nm ($R^2 = 0.9911$) in phosphate-buffered saline. Liposomal formulations of soya lecithin, DMPC, and DMPE were prepared. Initially, multilamellar vesicles were obtained. These vesicles were passed through polycarbonate filters (19 passes through 1 μm, 21 passes through 0.1 μm, respectively) in the mini-extruder to get large unilamellar vesicles. The size of the liposomes obtained after passing through the filter is shown in Table 1.

The entrapment efficiency was determined in the unilamellar vesicles of soyalecithin, DMPC and DMPE. Maximum entrapment was observed in DMPE liposomes. After determination of entrapment efficiency, the liposomes were subjected to transport studies across PAMPA and Caco-2 monolayer.

Permeation Studies using PAMPA. The average log P_e values for the formulations along with the standards propanolol, warfarin, and carbamazapine are given in Table 2. The log P_e value for serratiopeptidase, –7.72 cm/s (low

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Table 2. Log P_e Values from PAMPA Study

		lit. values[19]		
category	log P_e[a] (cm/s)	P_e	human absorption (%)	BCS permeability
pure drug	−7.72 ± 0.51			
soya lecithin liposome	−7.47 ± 0.36			
DMPC liposome	−7.01 ± 0.21			
DMPE liposome	−6.5 ± 0.35			
propanolol	−4.4 ± 0.12	−4.84	90	high
warfarin	−3.2 ± 0.26	−3.8	98	
carbamazepine	−5.1 ± 0.11	−5.21	100	high

[a] Average of three determinations.

Table 3. Apparent Permeability Values from Caco-2 Study

category	apparent permeability P_{app} (cm/s)[a]
pure drug	1.015×10^{-6}
soya lecithin liposome	1.43×10^{-6}
DMPC liposome	1.611×10^{-6}
DMPE liposome	1.245×10^{-6}
propanolol	9.1×10^{-6}
methotrexate	1.1×10^{-6}

[a] Average of three determinations.

permeation as per BCS classification[20]), considerably improved after formulation to –6.5, which is observed in the case of DMPE liposomes. The permeability ranking is as follows: DMPE liposome > DMPC liposome > soya lecithin liposome > pure drug. Thus, this is an indication that passive permeation of the drug has improved considerably on formulating it into liposomes.

Permeation Studies Using Caco-2. Prior to the start of the drug transport experiments, the TEER was measured in every well to confirm monolayer integrity. The TEER measurements for all experiments discussed here were typically in the range of 1504–1812 Ω. This range is indicative of successful formation of integral monolayer after 21 days of culture, which confirms differentiated monolayers with tight junctions. The Lucifer yellow passage was lowest (0.12–0.46%) for the 21 day culture, which further confirms differentiated monolayers with tight junctions.

The apparent permeability values obtained from the Caco-2 for the drug and the formulations calculated using eq 3 are given in Table 3. The apparent permeability increased significantly in all the formulations compared to the pure drug. Maximum apparent permeability was recorded for liposomes prepared from DMPC.

The cumulative amount of permeated drug was plotted against time and the slope of the linear part of the curve represented the steady-state flux rate. The permeability coefficient of the drug was calculated from the flux values. The permeation coefficient was found to be maximum in case of DMPC liposomes. Further, it could be seen that there is an increase in the flux and permeability in case of liposomal formulations when compared to the pure drug.

Table 4. Flux and Permeability Coefficient of Pure Drug and Formulations

category	flux (μg/cm^2/h)	permeation coefficient[a]
pure drug	3.208	2.955
soya lecithin liposome	4.535	4.176
DMPC liposome	4.955	4.563
DMPE liposome	3.888	3.582

[a] Average of three determinations.

Discussion

Transport of drug substances across the intestinal membrane is a complex and dynamic process. It includes the passage of compounds across various functional pathways in parallel. Passive permeability occurs through the cell membrane of enterocytes (transcellular) or via the tight junctions between the enterocytes (paracellular). Carrier-mediated transport occurs by the transport proteins present in the lipid membranes. Various influx (peptide transporters) and efflux mechanisms are also known in human intestine.

Phosphatidylcholines and phosphatidylethanolamines are neutral phospholipids carrying no net ionic charge. Lipid composition generally influences membrane fluidity, which can affect membrane permeability. Passive transcellular permeation is diffusion across a lipid bilayer and the permeability depends on the lipophilicity of the permeant. Hence, an improved permeation is seen with liposomal formulations.[21,22]

Liposomes showed entrapment efficiency greater than 50% in all cases. Extrusion of liposomes through polycarbonate membranes can give an apparent increase in encapsulation efficiency when the extrusions are carried out in the original mother liquid.[23] Thus, we could see that there was almost a 50% increase in entrapment efficiency on extrusion of the liposomes through the polycarbonate membranes. We have studied that the incorporation of cholesterol into liposomes can under appropriate conditions decrease the loss of trapped marker from liposome. This may be due to the ion−dipole interaction between lecithin and cholesterol probably not

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occurring to a significant extent.[24–26] Further, it may be due to the sterol promoting a tighter packing of the phospholipids and that the stability of the bilayer thereby increased due, in part, to greater van der Waals interaction between adjacent lipid molecules.[26]

Fick's law of diffusion clearly states that permeability is expected to be directly proportional to the buffer−membrane partition coefficient. Thus, either *%R* (mass balance calculated and expressed as percentage recovery) or P_e (effective permeability coefficient) may be used to represent lipophilicity.[17] The mass balance calculation determines the amount of drug retained by the artificial membrane or the Caco-2 monolayer. However, since P_e can be more reliably determined than *%R*, it is the preferred indicator of lipophilicity in this study.

PAMPA, a non-cell-based permeability model provides estimates of the passive transcellular permeability because it lacks transporter- and pore-mediated permeability. An adequate lipophilicity (log *P*) is required for a permeant to travel across the phospholipids membrane by passive diffusion.[27] Thus, it could be seen that when the liposomes were formulated log P_e values improved and hence the permeation. Caco-2 cells express pharmaceutically important drug transporters (e.g., PEPT1, OCT, OAT) though quantitatively under-expressed when compared with the in vivo situation. Caco-2 cells have significantly tight junctions that are significantly tighter compared with human intestine, and thus, Caco-2 cells normally underpredict the permeability value of drugs that are absorbed primarily via paracellular pathway. The apparent permeability values of the formulations were significantly greater for liposomal formulations when compared to the pure drug and the permeability was found to be best in case of DMPC liposomes.

A combination assay of PAMPA and unidirectional (apical to basal) Caco-2 permeability model can synergistically provide invaluable permeability/absorption assessment of the test compounds. An increase in both transcellular and paracellular permeation has been observed in case of the liposomal formulation of the drug serratiopeptidase. Thus, from the models studied, the use of liposomes proved as a potential oral delivery system for serratiopeptidase as there was improved oral absorption.

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