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Improved delivery of cromolyn from oral proliposomal beads

Deepali D. Deshmukh[a,*], William R. Ravis[a], Guru V. Betageri[b]

[a] 407 Walker Building, Harrison School of Pharmacy, Auburn University, Auburn, AL 36849, United States
[b] 309 E. Second Street, College of Pharmacy, Western University of Health Sciences, Pomona, CA 91766, United States

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ABSTRACT

Proliposomal bead formulations for improved oral delivery of cromolyn (BCS Class III compound) were formulated. Phospholipid (distearylphosphatidylcholine)–cholesterol-surfactant (Tween 80/sodium cholate) systems were spray-coated on beads containing cromolyn sodium and the dosage forms were characterized for vesicle formation and encapsulation efficiency. Delivery of cromolyn sodium from this novel dosage form was evaluated across the Caco-2 and everted rat intestinal sac model. Spontaneous formation of vesicles upon dilution of beads was observed. Enhancement in cromolyn transport was higher with phospholipids-surfactant proliposomal formulations compared to surfactant-free lipid formulations or pure surfactant solutions, most significant enhancement being with formulations with low surfactant concentration. No evidence of cellular damage to Caco-2 monolayers (e.g. significant decrease in the TEER) or change in transport and tissue accumulation of a marker molecule in the intestinal tissue model was observed. This indicated enhancement of transport via transcellular routes and not due to the modulation of the tight junctions or cell disruption. Results suggest that phospholipids-surfactant proliposomal beads offer a good potential for improved oral delivery of cromolyn.

1. Introduction

Cromolyn sodium is an anti-inflammatory drug used in prophylactic treatment of bronchial asthma and allergic rhinitis. Cromolyn is currently administered as an intranasal solution, powder or inhalation which is effective, but causes irritation at the dosing site. Cromolyn is poorly absorbed from the gastrointestinal tract (bioavailability < 1%) and it is a challenge to achieve therapeutically significant levels following oral administration. It has been suggested that due to its mast cell stabilizing effects, cromolyn might be useful in coronary artery disease (Kaartenin et al., 1994). In lieu of this, an oral dosage form that would deliver cromolyn in therapeutically active amounts is highly desirable. Prodrugs for oral administration of cromolyn have been synthesized in the past, but have met with limited success (Mori et al., 1988; Yoshimi et al., 1992). It has been suggested that increasing the lipophilic character of cromolyn could facilitate passive transport of the drug and, thereby, improve its absorption across the barrier membranes (Leone-Bay et al., 1996; Taylor et al., 1989). Liposomal encapsulation can be used to enhance lipophilicity; however, oral delivery of liposomes has met with limited success due to erratic and unpredictable absorption profiles (Dapergolas and Gregoriadis, 1976; Fukunaga et al., 1991; Arien et al., 1993; Chaudhari et al., 1994). The inability of liposomes to retain their integrity at the site of absorption is the major limiting factor for their successful utilization in oral delivery of drugs. One way to circumvent this problem is to formulate proliposomes as they offer the opportunity to form liposomes only at the site of delivery (Payne et al., 1986). A typical proliposomal system consists of a mixture of phospholipids and the active ingredient loaded onto a suitable carrier. Mixed micelles composed of phosphatidylcholines and surfactant can spontaneously form liposomes (vesicles) upon hydration with aqueous solution (Schurtenberger et al., 1985) and hence hold potential as a proliposomal system. Mixed micelles have been investigated as a proliposomal drug delivery system for water insoluble drugs (Son and Alkan, 1989; Supersaxo et al., 1991; Alkan-Onyuskel and Son, 1992; Sarbalouki et al., 2000). Despite their ability to enhance intestinal uptake of poorly absorbed drugs (Muranishi et al., 1979), the potential of lipid-based mixed micelles for delivery of small molecular weight hydrophilic drugs with poor absorption has not been investigated in detail.

This study intended to evaluate the utility of a proliposomal drug delivery system for cromolyn. We formulated phospholipid–cholesterol-surfactant-coated non-pareil beads, and evaluated their potential for proliposomal delivery. Non-pareil beads are inert pellets of uniform diameter made with sugar and starch. They are available in a range of sizes and can be used as starter cores for coating or layering. They provide a delivery system

* Corresponding author. Current address: Pharmaceutical Development, GlaxoSmithKline, 1250 S, Collegeville Road, Mail Code UP9200, Collegeville, PA 19406, United States. Tel.: +1 610 917 6514; fax: +1 610 917 4183.
E-mail address: deepali.d.deshmuhk@gsk.com (D.D. Deshmukh).

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resulting in high content uniformity, consistent and controlled drug release and good drug stability and hence were chosen as carriers for this system. The multiparticulate system thus achieved can be administered as capsules, sachets or tablets. Caco-2 cells were used to quantify cromolyn permeability and assess the toxicity profile of the formulations and the everted intestinal sac model was used to gain insight into the delivery mechanism of cromolyn from this novel dosage form.

2. Materials and methods

2.1. Materials

Cromolyn sodium, cholesterol, sodium cholate, polyoxyethylenesorbitan monooleate (Tween 80), Lucifer Yellow Salt (LY), Hanks Balanced Salts Solution (HANKS Buffer; HBSS) and Krebs–Hensleit Buffer were purchased from Sigma Chemical Company (St. Louis, MO). Distearylphosphatidylcholine (DSPC) was obtained from Avanti Polar Lipids (Birmingham, AL). Organic solvents (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ). The non-pareil beads (18/20 mesh) were obtained from Crompton and Knowles Ingredient Technology Corporation (Mahawah, NJ). Polyvinylpyrrolidone was a gift from ISP Technologies Inc. (Wayne, NJ). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Transwell® cell culture plates were from Costar (Cambridge, MA).

2.2. Preparation of proliposomal beads

The proliposomal beads were prepared using the conventional pan coating technique. Cromolyn, starch and talc were sieved through a #100 mesh screen. Required quantities (Table 1) of each ingredient were weighed and blended thoroughly in a V-blender. Polyvinylpyrrolidone (PVP) was dissolved in isopropyl alcohol (IPA) to give a 20% (w/v) solution. The coating pan was operated at 30–35 rpm. Non-pareil beads were placed in a pan and PVP solution was sprayed (1.5 g/min) onto the beads using a spray gun (Campbell Hausfeld, DH 7500). The powder blend was dusted so that the drug mix formed layers on the beads. An air pressure of 25 lb/in.^{2} was maintained throughout the coating process. This layering technique was continued until the entire powder blend was loaded onto the beads. The beads were dried overnight at room temperature and then sieved to remove any aggregates. To determine the loading efficiency a weighed amount of cromolyn-coated beads was diluted with phosphate-buffered saline (PBS). The samples were analyzed for cromolyn content using a reverse phase high pressure liquid chromatography (RP-HPLC) method described later in this section. The lipid and surfactant components were added to the beads using coating technique. The lipid composition was held constant for all the formulations. DSPC, cholesterol (in equimolar proportions), and the surfactant (Tween 80/sodium cholate) were weighed as required (Table 2) and were dissolved completely in a methylene chloride and isopropyl alcohol mixture (1:1) to obtain the desired lipid-to-surfactant ratios. The lipid solution was then coated (1 g/min) onto the dry drug loaded beads (cromolyn: DSPC molar ratio of 1:1) using the spray gun. The beads were dried for 24 h at room temperature. The lipid-coated beads were diluted with PBS and subjected to overnight extraction using a magnetic stirrer, and cromolyn content was determined using the analytical method described in Section 2.3.

Table 1
Composition of cromolyn-coated non-pareil beads

Ingredients	Quantity[a]
Cromolyn sodium (g)	2.5
Starch (g)	1.5
Talc (g)	2.5
PVP (20% solution in IPA) (mL)[b]	30

[a] Quantities per 100 g of non-pareil beads (#18 mesh).
[b] PVP, polyvinylpyroolidone; IPA, isopropyl alcohol.

Table 2
Composition of various proliposomal beads and test solutions

Composition	Code
Beads	
DSPC + cholesterol	Lipid
DSPC + cholesterol, Tween 80; molar ratio 1:0.3	I
DSPC + cholesterol, Tween 80; molar ratio 1:0.6	II
DSPC + cholesterol, Tween 80; molar ratio 1.0:1.2	III
DSPC + cholesterol, NaC; molar ratio 1:0.3	IV
DSPC + cholesterol, NaC; molar ratio 1:0.6	V
DSPC + cholesterol, NaC; molar ratio 1:1.2	VI
Solutions in HBSS/Krebs–Hensleit Buffer	
Cromolyn	Control
Tween 80 (control for I, II, III)	T-I/II/III
NaC (control for IV, V, VI)	NaC-I/II/III

2.3. HPLC analysis

Samples were analyzed for cromolyn content by RP-HPLC using the method described by Leone-Bay et al. (1996) with minor modifications with respect to the mobile phase. The HPLC system comprised a Star 9010 solvent system equipped with a Star 9095 variable wavelength UV–vis detector (Varian) connected to a Dynamax MacIntegrator. The mobile phase consisted of 0.067 M potassium phosphate buffer, acetonitrile, and triethanolamine (75:25:0.02) set at a flow-rate of 1.0 mL/min. The chromatograms were eluted on a Phenomenex LUNA C-18(2) (5 μm, 150 mm × 4.6 mm) column at a wavelength of 220 nm. For the samples from the intestinal sac study (Section 2.7) LY was measured simultaneously with cromolyn.

2.4. Formation of vesicles in situ

2.4.1. Encapsulation efficiency

The encapsulation efficiency, defined as the percentage of cromolyn encapsulated in the vesicles, was determined by the protamine-induced aggregation method (Kulkarni et al., 1995). Briefly, the beads were disintegrated in PBS and allowed to equilibrate at room temperature to yield a lipid concentration of approximately 10 mg/mL. To 250 μL of the preparation, an equal quantity of protamine solution in PBS (50 mg/mL) was added and vortexed for 2 min and the mixture was then incubated for 12 h at room temperature. After incubation, the mixture was centrifuged at 12,000 rpm for 5 min. The supernatant was separated and the pellet washed with 100 μL of PBS and centrifuged for 5 min. The quantity of cromolyn sodium in the pellet and supernatant was determined by RP-HPLC analysis.

2.4.2. Particle size analysis

Samples obtained after reconstitution of the beads were characterized for their size distribution using a Nicomp Submicron Particle Sizer (Model 370). The measurements were made (as volume diameter) at 23 °C and a viscosity of 1.333 cP. In addition, the samples were subjected to transmission electron microscopy to evaluate bilayer/vesicle formation.

2.5. In vitro dissolution study

In vitro dissolution studies were performed on the proliposomal beads using the conventional USP II dissolution apparatus (SR-2,

Hanson Research, Northridge, CA) operated at a paddle speed of 50 rpm. 500 mg of the beads were added to the dissolution flask containing 500 mL of PBS (pH 7.4) maintained at 37 °C ± 1 °C. 3 mL samples were withdrawn at pre-determined intervals and replaced with fresh buffer. The samples were filtered through a 0.45 μm filter and analyzed by RP-HPLC.

2.6. Caco-2 cell study

2.6.1. Transport study

Caco-2 cells, 17 days old, seeded into a 12 well plate with polyester treated 0.4 μm inserts with a surface area of 1 cm^2 Transwell® inserts were used for transport studies. The beads were suitably reconstituted and equilibrated using HBSS prior to yield cromolyn concentration of 250 μg/mL. Standard surfactant solutions at concentrations corresponding to the formulation were used as controls (Table 2). 500 μL of the test suspension/solution was added to the donor compartment and 1.5 mL of HBSS was added to the receiver compartment. Samples (300 μL) were withdrawn at fixed intervals and replenished with an equal amount of HBSS. The samples were analyzed for cromolyn sodium content by RP-HPLC. Flux (μg/min) from apical to basolateral side was determined by regression analysis of the plots.

2.6.2. Measurement of transepithelial resistance (TEER)

The integrity of the cell monolayers at different times was monitored by measuring the transepithelial electrical resistance (Ω cm^2) (TEER) using a voltmeter (EVOM®, World Precision Instruments, Sarasota, FL). For TEER measurements, the cell monolayers were removed from the incubator and the TEER was measured in a laminar flow hood. The TEER of the Caco-2 cell monolayers in the presence of HBSS was found to be in the range of 440 ± 64 (Ω cm^2) and was used as a control value for all the studies.

2.7. Everted intestinal sacs study

2.7.1. Preparation of the test solutions

Test solutions were prepared similar to those for the Caco-2 cell study (Table 2), except HBSS was substituted with Krebs–Hensleit Buffer (pH 7.4) and Lucifer Yellow (LY; molecular weight 457.3), a probe for intestinal viability and intercellular transport (Mineo et al., 2002), was added to all the test solutions. Formulation I and IV (showed maximum cromolyn transport in Caco-2 cells) and formulations III and VI (showed least enhancement of cromolyn transport in Caco-2 cells) were chosen for this study.

2.7.2. Preparation of the intestinal sacs and uptake studies

Everted intestinal sac experiments were performed according to the method described by Ravis and Feldman (1978). The animal work carried out during the course of research has been approved by the Auburn University Animal Care and Use Committee. Healthy male Sprague–Dawley (Harlan, Indianapolis, IN) rats (225–250 g) were fasted overnight. On the day of the experiment, a rat was anaesthetized with isoflurane in a glass chamber in a vented hood. After the rat showed no response to pain, a midline abdominal incision was made and the entire length of intestine was quickly removed. The first 15 cm segment distal to the pylorus was discarded. The excised intestine was flushed with ice-cold normal saline to remove any intestinal contents. The intestinal segment was everted on a thin glass rod. The everted intestine was gently stretched by a 2 g weight and cut into four segments (10 cm long). Each of the sacs was mounted individually in a perfusion apparatus consisting of a 100 mL glass tube (35 mm diameter) fitted with a conical rubber stopper. The rubber stopper housed one port for removal and addition of the serosal fluid. Another port on the stopper maintained a continuous supply of 5% CO_2 and 95% O_2 during the entire length of the experiment. The perfusion apparatus was filled with 60 mL of the test solution, which served as the mucosal fluid (outer compartment) for the experiment. All the test solutions were prepared in Krebs–Hensleit Buffer (final pH 7.4). Experiments were performed at 37 ± 0.5 °C in a water bath.

After introduction of the intestinal sacs into the perfusion chamber, 2 mL of Krebs–Hensleit Buffer (pre-saturated with 5% CO_2 and 95% O_2) were introduced into the intestine generating a serosal fluid. All the fluid from the serosal compartment was drawn into a syringe with thin rubber tubing at 10 min intervals for a 40 min period. Fresh buffer was added to the inner chamber after every sampling. The final volume of the sample was adjusted to 4 mL with fresh Krebs–Hensleit Buffer and all the samples were stored in marked and stoppered centrifugation tubes until the time of analysis. The viability of the intestinal membrane during the course of the experiments was monitored by measuring the transport of Lucifer Yellow. Cromolyn, and the LY content in the samples was measured simultaneously by RP-HPLC.

2.7.3. Measurement of cromolyn and LY in the serosal fluid

500 μL of the serosal fluid collected at each time point was used for determination of cromolyn and LY content. All samples were analyzed by RP-HPLC using the method described before. The amount of cromolyn and LY transported from the mucosal to the serosal side was calculated and plotted against time. The flux (μg/min) was determined by regression analysis of the plots.

Ultracentrifugation has been used to determine presence of intact liposomes in transport studies with intestinal models (Rowland and Woodley, 1981a,b,c). Serosal fluid samples collected for each time-point in individual treatments were pooled and centrifuged at 5000 × *g* for 10 min and then analyzed for cromolyn content. Total cromolyn content and amount of non-encapsulated cromolyn was determined for each treatment.

2.8. Statistical analysis

The data were analyzed to estimate significant differences between the formulations using SAS for Windows by SAS Institute Inc. (Cary, NC). All the comparisons were made using the Tukey's Studentized Range (HSD) with a minimal sample size of three for each study (significance: $^{*}p < 0.05$).

3. Results

3.1. Formation and characterization of liposomes

The encapsulation efficiency, expressed as percent of the initial amount, for various formulations was calculated (Table 3). The

Table 3
Particle size and encapsulation efficiency of various proliposomal bead formulations

Formulation	Particle size (nm)	Encapsulation (%)
Lipid	1070 (179.6)	65.2 (2.4)
I	460 (78)	45.1 (0.6)
II	22.8 (2.9), 3015 (507)	15.4 (3.2)
III	[a]	[b]
IV	574 (93)	29.4 (6.8)
V	24.5 (2.9), 2154 (360)	9.6 (4.2)
VI	[a]	15.1 (4.9)

Values expressed as mean (S.D.); $n = 3$ for particle size and $n = 4$ for encapsulation (%).

[a] Highly polydisperse samples.
[b] Amount below limit of quantification of the HPLC assay system.

encapsulation of cromolyn was highest in the absence of surfactants and decreased with increasing amounts of surfactants. Higher encapsulation with Tween 80 in comparison to sodium cholate indicates that the extent of encapsulation is dependent on both the type and amount of the surfactant employed. Table 3 shows the particle size distribution of the samples obtained upon reconstitution of the beads. Uniform size distribution was observed at lower surfactant concentrations, whereas the particles were non-homogenous at higher concentrations of surfactants.

TEM micrographs of the formulations with varied surfactant content are shown in Fig. 1. Vesicle formation could be seen upon dilution of the proliposomal beads. Fig. 1c shows a bi-modal size distribution in formulations II and V. Formation of intermingled mesh-like vesicles explains the polydispersity in formulations containing a higher surfactant concentration (Fig. 1d).

3.2. In vitro dissolution studies

Fig. 2A and B shows the dissolution profile of cromolyn from the proliposomal bead formulations. The rate of release of cromolyn from the beads was slower and biphasic in the lipid formulation. Cromolyn release from formulation I also exhibited a biphasic release, a pattern characteristic to liposomes (Kulkarni et al., 1997), with almost 60% of release within 90 min and complete drug release by the end of 2 h. In formulations II and III, the entire amount of cromolyn was released within the first 20 min. Unlike the formulations with Tween 80, cromolyn release was rapid in formulations IV, V, VI.

3.3. Caco-2 cell studies

Fig. 3A and B shows cromolyn transport from various formulations in Caco-2 cell model. It is clear from the enhancement in cromolyn transport (Fig. 3) followed a linear trend. Increased transport without a lag time was in line with the dissolution of the formulations and was as expected. Transport of cromolyn from the phospholipids-surfactant formulations varied with the type and amount of surfactant. Table 4 compares the flux of cromolyn from formulations with the values obtained with surfactant solutions and the phospholipid–cholesterol formulation. Formulations I and IV caused 7- and 4-fold increase in the flux respectively. The surfactant solutions affected flux at higher concentrations; the extent of increase was, however, lower compared to the formulation I and I.

In order to gain insight into the mechanism of permeation enhancement from formulations and evaluate their toxicity profile, TEER, a qualitative measure of paracellular permeability (Meaney and O'Driscoll, 2000), was monitored throughout the transport experiments. TEER profiles of the Caco-2 cell monolayers in the presence of test solutions have been presented in Fig. 4A and B. Surfactant solutions affected the TEER in a concentration-dependent manner whereas TEER remained unchanged with lipid-based formulations. Higher flux observed with formulations I and IV without a significant change in the TEER suggest that higher permeation was not due to modulation of the tight junctions and or a compromised membrane, but possibly due to transcellular pathways.

3.4. Everted intestinal sac studies

Fig. 5A and B show cromolyn transport from various formulations in the intestinal sac model. Based on the cumulative amount of cromolyn transported over time, flux (μg/min), a parameter independent of the surface area, was calculated as a measure of permeation enhancement. Among the lipid-based formulations, the order of enhancement was I > IV > lipid > VI > control (Table 4). In

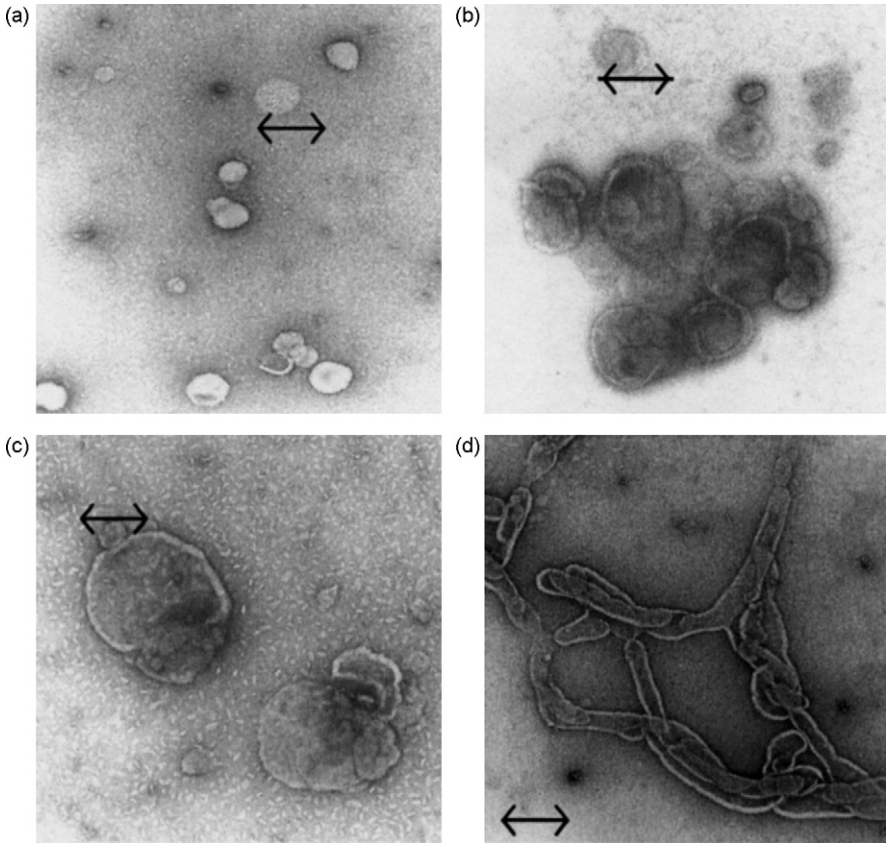

Fig. 1. Transmission electron micrographs of vesicles derived from cromolyn formulations in PBS buffer (pH 7.4). Bar equals 0.5 μm. (a) I, (b) IV, (c) V, and (d) VI.

Table 4
A comparison of cromolyn transport (expressed as flux) in Caco-2 cells and intestinal sacs

Formulation	Caco-2 cells		Intestinal sacs	
	Flux × 10^{-4} (μL/min)	Enhancement ratio	Flux (μL/min)	Enhancement ratio
HBSS (control)	7.8 (1.05)	1	–	–
Krebs–Hensleit Buffer (control)	NA	–	0.173 (0.067)	1
Lipid	17.9* (2.6)	2.3	0.268* (0.017)	1.5
I	57.6* (2.4)	7.4	0.607* (0.019)	3.5
T-I	10.8 (2.3)	1.4	0.254* (0.029)	1.5
II	17.4 (5.3)	2.2	Not tested	–
T-II	21.8* (6.2)	2.8	Not tested	–
III	16.7 (6.3)	2.1	0.167 (0.094)	1
T-III	21.9* (6.5)	2.8	Not tested	–
IV	31.4* (7.1)	4	0.421* (0.013)	2.4
NaC-I	17.3* (0.9)	2.2	0.291* (0.176)	1.7
V	19.7* (4.4)	2.5	Not tested	–
NaC-II	17.4* (3.5)	2.2	Not tested	–
VI	15.6 (0.5)	2	0.224 (0.123)	1.3
NaC-III	19.2* (2.3)	2.5	Not tested	–

* $p < 0.05$.
Values are expressed as mean (S.D.); $n = 4$.

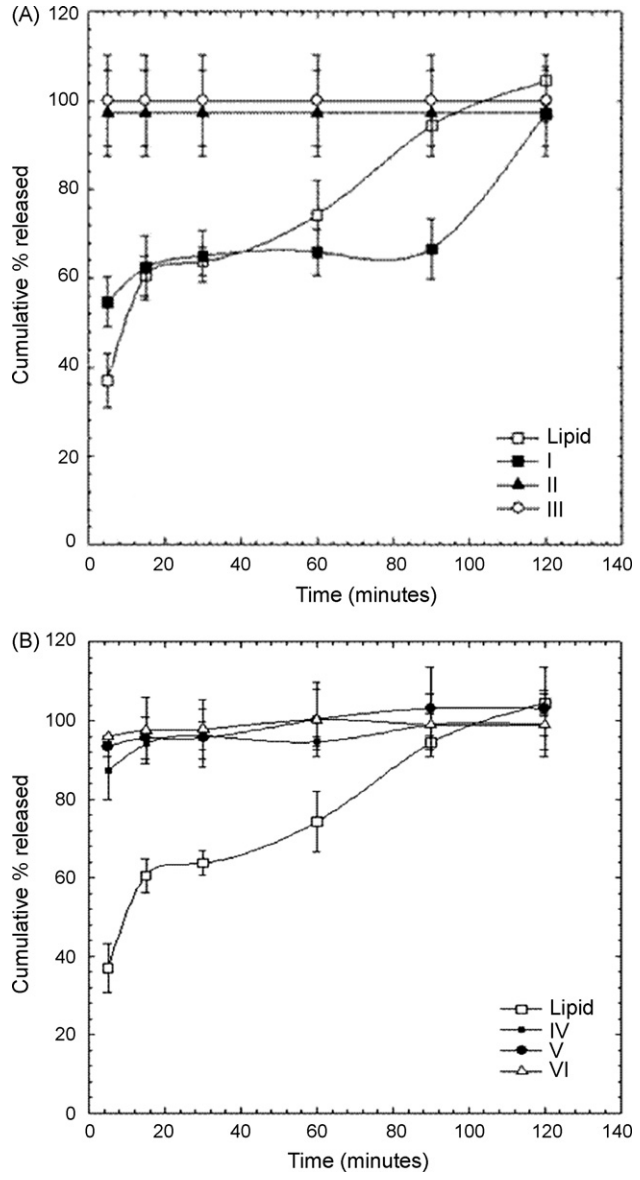

Fig. 2. Dissolution profile of cromolyn from Tween 80 (A) and Sodium cholate (B) proliposomal formulations.

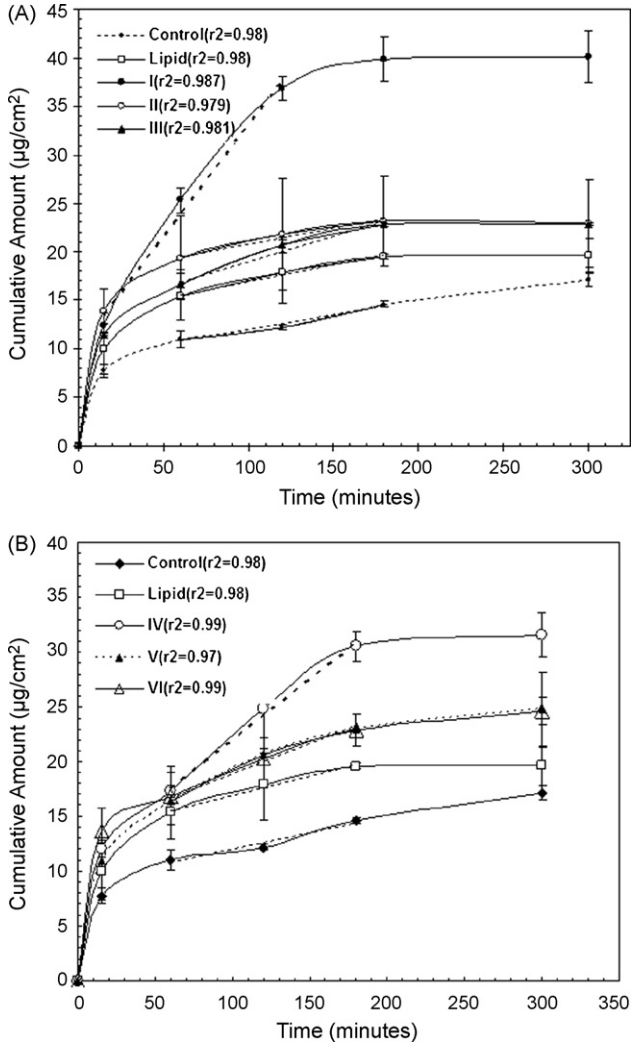

Fig. 3. Cromolyn transport from Tween 80 (A) and sodium cholate (B) formulations across Caco-2 cells.

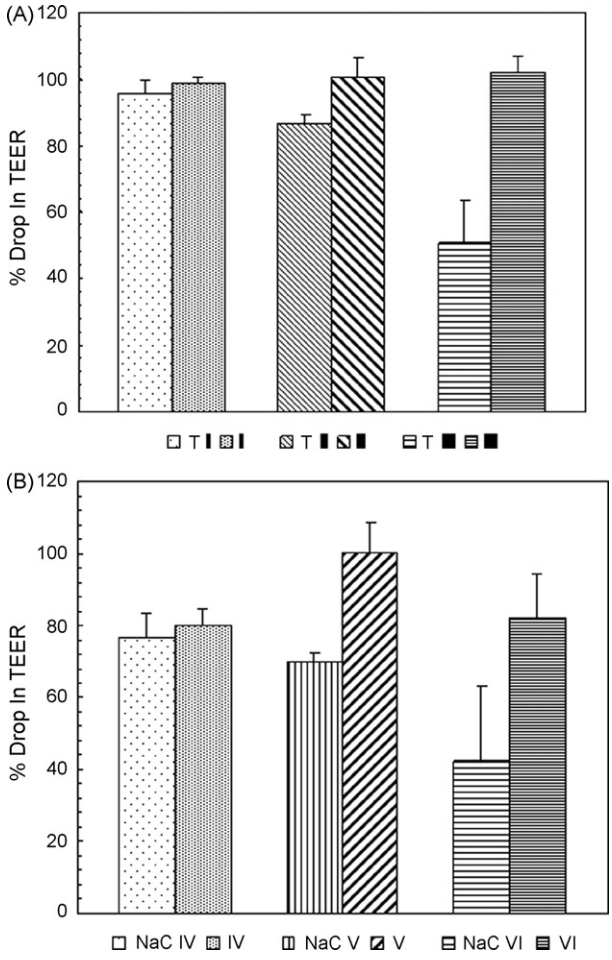

Fig. 4. TEER (% of control) in Caco-2 cells with Tween 80 (A) and sodium cholate (B) formulations.

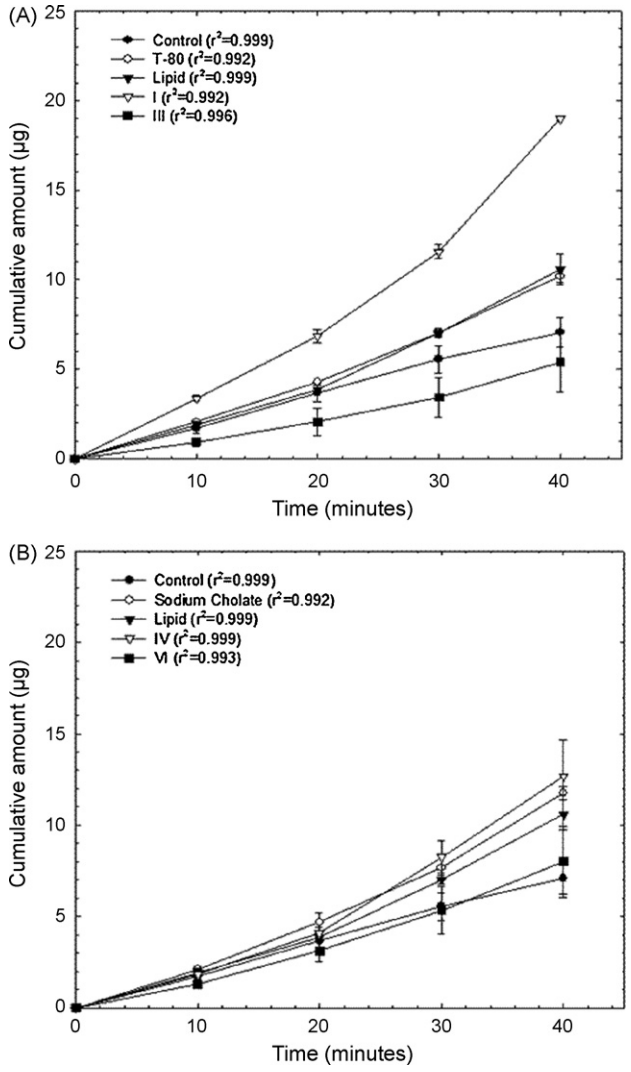

Fig. 5. Cromolyn transport from Tween 80 (A) and sodium cholate (B) formulations in intestinal sacs.

agreement with the observations in the Caco-2 cell model, formulation III did not have a significant affect on cromolyn transport. Measurement of LY accumulation showed no significant increase in transport in the presence of formulations (lipid, I and IV, Fig. 6), the LY accumulation, however, was greater with Tween 80, NaC and formulations III and IV.

Analysis of the serosal samples following ultracentrifugation indicated that cromolyn existed predominantly in the free form thereby making the amount of liposome-encapsulated cromolyn in the serosal fluid, insignificant (Table 5).

4. Discussion

The main objective of this study was to determine the feasibility of using proliposomal beads for improved oral delivery of cro-

Table 5
Centrifugation of serosal contents from the everted intestinal sacs indicated absence of intact liposomes

Test solution	Cromolyn (µg)		Change (%)
	Before centrifugation	After centrifugation	
Control	7.1 (0.8)	7.4 (0.5)	4.2
Lipid	9.2 (0.9)	9.1 (0.7)	1.1
I	18.9 (0.1)	19.2 (0.2)	1.6
III	5.4 (1.7)	6.0 (1.6)	1.1
IV	12.7 (0.9)	11.6 (1.3)	8.7
VI	8.0 (1.9)	7.2 (0.3)	10.0

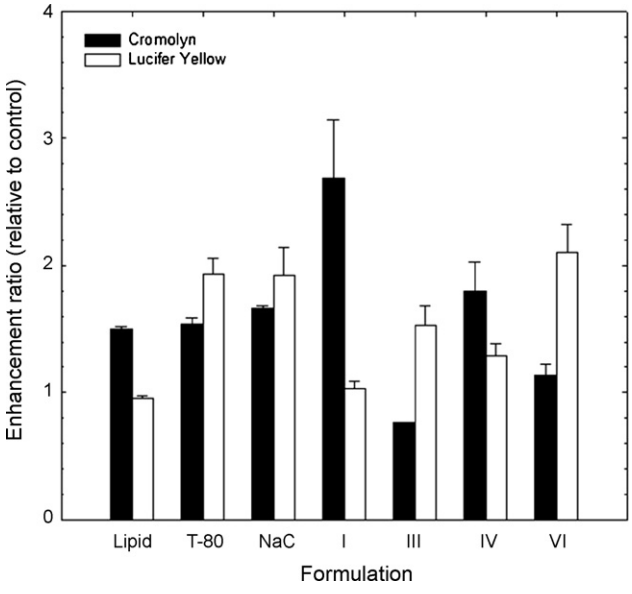

Fig. 6. Effect of test solutions on cromolyn and LY accumulation in intestinal sacs.

molyn. The encapsulation efficiency of the formulation and effects on cromolyn permeability was therefore studied. We formulated and evaluated non-pareil beads coated with lipid and two types of surfactant at varying molar ratios. Distearoylphosphatidylcholine (DSPC), a phospholipid with a high phase transition temperature and better stability in the GI environment compared to other phospholipids, especially in the presence of bile salts (Rowland and Woodley, 1980), was chosen as the lipid component for formulation. Tween 80 and sodium cholate were chosen as the surfactants for this study, whereas cholesterol was added to the lipid phase due to its ability to enhance encapsulation of hydrophilic drugs and improve stability of the vesicles (Betageri, 1993). Extent of enhancement of cromolyn transport from these formulations was measured in Caco-2 cells and everted rat intestinal sacs models.

The experiments carried out to characterize the physical properties of the formulations showed that the beads formulated in this study were able to produce vesicles *in situ*. The formation of liposomes as described by calculation of encapsulation efficiency and particle size analysis depended largely on the lipid-to-surfactant ratio. Formulations with lowest surfactant content showed maximum encapsulation (formulations I and IV) and the most enhancement in cromolyn transport. However, at the same ratio, encapsulation was higher with Tween 80 (formulation I) compared to sodium cholate (formulation IV). An increase in encapsulation of estradiol in dipalmitoylphosphotidylcholine liposomes with decreasing Tween 80, Span 80 and sodium cholate concentration has been reported in the past (El Maghraby et al., 2000). Higher encapsulation with Tween 80 formulations as compared to sodium cholate formulations was also consistent with this work. Increased encapsulation with Tween 80 could be due to the beneficial effect of Tween 80 on the size and stability of liposomes, which has been well documented in literature (Kronberg et al., 1990; Zou et al., 1996). The mechanism of formation of vesicles from mixed micelles begins by dilution of mixed micelles followed by progressive depletion of surfactant until vesicles only contain lipid molecules (Olivion et al., 2000). The absence of micelle to vesicle transition due to insufficient removal of surfactant could be a possible cause for negligible encapsulation in formulations with higher surfactant content. The presence of mesh like mixed vesicular structures and micelles as seen from TEM and particle size distribution measurements further corroborate the non-existence of lipid vesicles at higher concentrations of the surfactant. An alternative cause for lower encapsulation at high surfactant concentrations, especially sodium cholate, could be partial solubilization of liposomes leading to co-existence of mixed micelles and vesicles as reported in the literature (Almog et al., 1986). The rate of release of cromolyn as seen from the dissolution profiles varied with the extent of encapsulation. In agreement with published literature (Kokkona et al., 2000), cromolyn release from formulations with high encapsulation efficiency was prolonged. Liposomes are known to extend the release of their contents over an extended period of time. However, we observed instantaneous release of cromolyn from formulations with high surfactant concentrations. Low encapsulation could be the reason for this rapid release; however, marginally extended release (2 h) from formulations with higher encapsulation was not expected. Experimental methods employed to determine release of encapsulated material from conventional liposomes entail incubation of liposomes with small amounts of buffer (Kokkona et al., 2000) or use of dialysis membranes (Kulkarni et al., 1997; Dipali et al., 1996). In an attempt to mimic realistic conditions, release of cromolyn from the formulations was studied by the dissolution method recommended for conventional solid dosage forms. The rate and extent of release of the encapsulated material from liposomes increases with agitation (Vemuri and Rhodes, 1995), which could be the reason for rapid release of cromolyn from encapsulated material.

In vitro transport studies in models revealed that formulation with high encapsulation efficiency showed increased cromolyn permeability. In contrast to formulations I and IV, the increase in cromolyn transport with formulations II, III, V and VI was marginal (Table 3) indicating that surfactant concentration is critical to permeation enhancement. El Maghraby and co-workers (2000) have reported the importance of optimum surfactant concentration for maximum delivery of estradiol from lipid vesicles, observations from the present study further highlight the importance of surfactant concentration for improved delivery from lipid-based formulations.

Inclusion of phospholipid with surfactant resulted in protective action on the cell monolayers (as evidenced by constant TEER), which has been reported in the past (Velardi et al., 1991). Transport studies in *in vitro* models did exhibit an increase in cromolyn transport with lipid formulation, but the extent of permeability was lower than formulations with surfactants (I and IV). Werner and co-workers (1996) did not observe any improvement in transport of CRC 220, a hydrophilic peptidomimetic thrombin inhibitor, with phosphatidylcholine:bile salt or cholesterol:bile salt mixed micelles. Surfactants in the presence of lipids are known to act as edge activators making the liposome structure highly deformable (Planas et al., 1992; Cevc et al., 1993). The high deformability of these vesicles enhances drug delivery across barriers (Cevc et al., 1995). DSPC is known to form relatively stable and rigid liposomes due to its high phase transition temperature (Kulkarni et al., 1995). Formulation of cromolyn with DSPC and cholesterol results in more "rigid" liposomes, whereas addition of a surfactant makes the liposomes more "flexible". This "flexibility" or "deformability" could have rendered formulation I and IV conducive to cellular uptake as opposed to liposomes without surfactant.

It is generally assumed that small, hydrophilic drug molecules are transported across the intestinal membranes mainly via the paracellular pathway (Artursson et al., 2001). Transport of cromolyn across Caco-2 cells has not been studied in detail before. However, its high water solubility and relatively small molecular weight (512.3) makes it a suitable candidate for paracellular transport. The entry of molecules through this route is primarily restricted by tight junctions and, hence, loosening of these junctions offers a pathway to enhance paracellular transport (Hochman and Artursson, 1994). Bile salts and synthetic surfactants are known to enhance uptake of paracellularly transported drugs (Werner et al., 1996; Aungst, 1993) and hence surfactant-related change in cromolyn permeation was expected. In the Caco-2 cells, higher surfactant concentrations led to an increase in cromolyn permeation; however, it was accompanied by a significant drop in TEER values (Fig. 4A and B). In contrast formulations I and IV were able to significantly enhance cromolyn transport without affecting the TEER. This suggests that the increased permeability from the formulations was not a result of membrane damage or modulation of the tight junctions.

The intestinal sac model, both LY and cromolyn showed increased serosal accumulation in the presence of pure surfactant whereas lipid and formulations I and IV exclusively improved cromolyn uptake (Fig. 6). Being a paracellularly transported molecule, enhancement of LY transport would involve manipulation of intercellular tight junctions or transport via a damaged membrane. The absence of improvement in LY uptake by I, IV or the lipid treatments suggests that these formulations do not affect the tight junctions or the membrane integrity. On the other hand a higher enhancement ratio of LY was observed with III, VI. Enhanced transport of LY without a change in cromolyn transport was seen with formulations III, VI and surfactant solutions. Surfactant solutions are known to improve uptake of certain compounds due to increase in paracellular transport, which explains increased LY transport with surfactant

solutions, III, and IV (formulations with low encapsulation of cromolyn, higher concentration of surfactants). This indicates that a transport of a marker molecule like LY could be enhanced via paracellular modulation, however, that approach was not effective to enhance cromolyn uptake. The enhancement in cromolyn transport is truly a "formulation-effect". Being a paracellularly transported molecule, enhancement of LY transport would involve manipulation of intercellular tight junctions or transport via a damaged membrane. The absence of improvement in LY uptake by I, IV or the lipid treatments suggests that these formulations do not affect the tight junctions or the membrane integrity.

Analysis of the serosal fluid samples from the intestinal sac study showed that cromolyn was transported to the serosal side in the non-encapsulated form and no evidence of transport of intact liposomes was observed. Upon dilution, formulations I and IV formed vesicles with diameters ranging from 400 to 600 nm. The apparent radius of the junctional pore in the Caco-2 cell model being in the range from 8 to 12 nm (Adson et al., 1994; Meaney and O'Driscoll, 2000), making the transport of intact liposomes a formidable challenge. Observations from the *in vitro* models evaluated in this study indicate that enhanced cromolyn transport with formulations I and IV, was due to uptake via an intact cell membrane and not due to transfer of intact liposomes.

In the present study, we have been able to demonstrate systematically, the usefulness of proliposomes to enhance delivery of cromolyn across Caco-2 cell monolayers and the intestinal membrane. We have demonstrated that vesicles can be formed *in situ* upon hydration of the beads formulated in this study. Addition of surfactant rendered the vesicles more flexible, the extent of cromolyn encapsulation and permeation was dependent on the type of surfactant and the lipid-to-surfactant ratio. The enhanced transport appears to be due to uptake of liposomes through an intact cell membrane; however, further studies are required to identify the exact mechanism. The free-flowing beads formulated in this study could be used for modified release (by functional coating) or conveniently administered as enteric-coated capsules for site-specific delivery. Enhanced cromolyn transepithelial flux observed in this study (4–7-fold) warrants further investigation in humans. An understanding of the clinical relevance of the *in vitro* data will be very valuable in understanding the commercial viability of the proliposomal bead dosage form. Formulation design of proliposomal beads with other phospholipids and usefulness of this dosage form for delivery of other drug substances is being evaluated in our laboratory.

References

Adson, A., Raub, T.J., Burton, P.S., Barsuhn, C.L., Hilgers, A.R., Audus, K.L., Ho, N.F.H., 1994. Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers. J. Pharm. Sci. 83, 1529–1536.

Alkan-Onyuskel, H., Son, K., 1992. Mixed micelles as proliposomes for the solubili zation of teniposide. Pharm. Res. 9, 1556–1662.

Almog, S., Kushnir, T., Nir, S., Lichenberg, D., 1986. Kinetic and structural aspects of reconstitution of phophatidylcholine vesicles by dilution of phosphatidylcholine-sodium cholate mixed micelles. Biochemistry 25, 2597–2605.

Arien, A., Goigouux, C., Baquey, C., Dupuy, B., 1993. Study of *in vitro* and *in vivo* stability of liposomes loaded with calcitonin or indium in the gastrointestinal tract. Life Sci. 53, 1279–1290.

Artursson, P., Palm, K., Luthman, K., 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Deliv. Rev. 46, 27–43.

Aungst, B.J., 1993. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. J. Pharm. Sci. 82, 979–987.

Betageri, G.V., 1993. Liposomal encapsulation and stability of dideoxyinosine triphosphate. Drug Dev. Ind. Pharm. 19, 531–539.

Cevc, G., Schatzlein, A., Gebauer, D., Blume, G., 1993. Ultra-high efficiency of drug and peptide transfer through intact skin by menas of novel drug-carriers, transferosomes. In: Brain, K.R., James, V.A., Walters, K.A. (Eds.), Prediction of Percutaneous Penetration, 3rd International Conference, 14–16 April, vol. 3b. STS Publishing, Cardiff, UK, pp. 226–236.

Cevc, G., Schatzlein, A., Blume, G., 1995. Transdermal drug carriers: basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. J. Control. Rel. 36, 3–16.

Chaudhari, K.B., Labhasetwar, V., Dorle, A.K., 1994. Liposomes as a carrier for oral administration of insulin: effect of formulation factors. J. Microencapsul. 11, 319–325.

Dapergolas, G., Gregoriadis, G., 1976. Hypoglycaemic effect of liposome entrapped insulin administered intragastrically into rats. Lancet 2, 824–827.

Dipali, S.R., Kulkarni, S.B., Betageri, G.V., 1996. Comparative study of separation of non-encapsulated drug from unilamellar liposomes by various methods. J. Pharm. Pharmacol. 48, 1112–1115.

El Maghraby, G.M.M., Williams, C.A., Barry, B.W., 2000. Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. Int. J. Pharm. 196, 63–74.

Fukunaga, M., Miller, M.M., Deftos, L.J., 1991. Liposome-entrapped calcitonin and parathyroid hormone are orally effective in rats. Horm. Metab. Res. 23, 166–167.

Hochman, J., Artursson, P., 1994. Mechanisms of absorption enhancement and tight junction regulation. J. Con. Rel. 29, 253–267.

Kaartenin, M., Pentilla, A., Kovanen, P.T., 1994. Mast cells of two types differing in neutral protease composition in the human aortic intima. Arterioscler. Thromb. 14, 966–972.

Kokkona, M., Kallinteri, P., Fatouros, D., Antimisiaris, S.G., 2000. Stability of SUV liposomes in the presence of cholate salts and pancreatic lipases: effect of lipid composition. Eur. J. Pharm. Sci. 9, 245–252.

Kronberg, B., Dahlman, A., Carlfors, J., Karlsson, J., Artursson, P., 1990. Preparation and evaluation of sterically stabilized liposomes: colloidal stability, serum stability, macrophage, uptake, and toxicity. J. Pharm. Sci. 79, 667–671.

Kulkarni, S.B., Betageri, G.V., Singh, M., 1995. Factors affecting microencapsulation of drugs in liposomes. J. Microencap. 12, 229–246.

Kulkarni, S.B., Singh, M., Betageri, G.V., 1997. Encapsulation, stability and *in vitro* characteristics of lipososmal formulations of Colchicine. J. Pharm. Pharmacol. 49, 491–495.

Leone-Bay, A., Leipold, H., Sarubbi, D., Variano, B., Rivera, T., Baughman, R.A., 1996. Oral delivery of sodium cromolyn: preliminary studies *in vivo* and *in vitro*. Pharm. Res. 13, 222–225.

Meaney, C.M., O'Driscoll, 2000. A comparison of the permeation enhancement potential of simple bile salt and mixed bile salt:fatty acid micellar systems using Caco-2 cell culture model. Int. J. Pharm. 207, 21–30.

Mineo, H., Hara, H., Shigematsu, N., Okuhara, Y., Tomita, F., 2002. Melibiose, Difructose Anhydride III and Difructose Anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions *in vitro*. Nut. Interact. Tox. 132, 3394–3399.

Mori, T., Nishimura, K., Tamaki, S., Nakamura, S., Tsuda, H., Kakaya, N., 1988. Prodrugs for the oral delivery of disodium cromoglycate. Chem. Pharm. Bull. 36, 338–344.

Muranishi, S., Muranishi, N., Sezaki, H., 1979. Improvement of absolute bioavailability of normally poorly absorbed drugs: inducement of the intestinal absorption of streptomycin and gentamycin by lipid-bile salt mixed micelles in rat and rabbit. Int. J. Pharm. 2, 101–111.

Olivion, M., Lesieur, S., Gabrielle-Madelmont, C., Paternostre, M., 2000. Vesicle reconstitution from lipid-detergent mixed micelles. Biochim. Biophys. Acta 1508, 34–50.

Payne, N.I., Timmins, P., Ambrose, C.V., Ward, M.D., Ridgway, F., 1986. Proliposomes: a novel solution to an old problem. J. Pharm. Sci. 75, 325–329.

Planas, M.E., Gonzalez, P., Rodriguez, L., Sanchez, G., 1992. Noninvasive percutaneous induction of topical analgesia by a new type of drug carrier and prolongation of local pain insensitivity by anesthetic liposomes. Anest. Analg. 75, 615–621.

Ravis, W.R., Feldman, S., 1978. Effect of enzyme-inducing and enzyme-inhibiting agents on drug absorption I: 3-O-methylglucose transport in rats. J. Pharm. Sci. 67, 245–248.

Rowland, R.N., Woodley, J.F., 1980. The stability of liposomes *in vitro* to pH, bile salts and pancreatic lipase. Biochim. Biophys. Acta 620, 400–409.

Rowland, R.N., Woodley, J.F., 1981a. The uptake of distearoylphosphatidylcholine/cholesterol liposomes by rat intestinal sacs *in vitro*. Biochim. Biophys. Acta 673, 217–223.

Rowland, R.N., Woodley, J.F., 1981b. Uptake of free and liposome-entrapped insulin by rat intestinal sacs *in vitro*. Biosci. Rep. 1, 345–352.

Rowland, R.N., Woodley, J.F., 1981c. Uptake of free and liposome-entrapped horseradish peroxide by rat intestinal sacs *in vitro*. FEBS Lett. 123, 41–44.

Sarbalouki, M.N., Parsaee, S., Kosary, P., 2000. Mixed micelle proliposomes for preparation of liposomes containing amphotericin B, *in vitro* and *ex vivo* studies. PDA J. Pharm. Sci. Tech. 54, 240–246.

Son, K., Alkan, H., 1989. Liposomes prepared dynamically by interactions between bile salts and phospholipid molecules. Biochim. Biophys. Acta 981, 288–294.

Schurtenberger, P., Mazer, N., Kanzig, W., 1985. Micelle to vesicle transition in aqueous-solutions of bile-salt and lecithin. J. Phys. Chem. 89, 1042–1049.

Supersaxo, A., Hein, W.R., Steffen, H., 1991. Mixed micelles as a proliposomal lymphotropic drug carrier. Pharm. Res. 8, 1286–1291.

Taylor, K.M., Taylor, G., Kellaway, I.W., Stevens, J., 1989. The influence of liposomal encapsulation on sodium cromoglycate pharmacokinetics in man. Pharm. Res. 6, 633–636.

Velardi, A.L.M., Groen, A.K., Elferink, R.P.J.O., van der Meer, R., Palasciano, G., Tygat, G.N.J., 1991. Cell type-dependent effect of phospholipids and cholesterol on bile salt toxicity. Gastroentorology 101, 457–464.

Vemuri, S., Rhodes, C.T., 1995. Development and validation of a drug release rate method for a water soluble drug in a liposome preparation. Drug Dev. Ind. Pharm. 21, 1353–1364.

Werner, U., Kissel, T., Reers, M., 1996. Effects of permeation enhancers on the transport of a Peptidomimetic Thrombin Inhibitor (CRC 220) in a human intestinal cell line (Caco-2). Pharm. Res. 13, 1219–1227.

Yoshimi, A., Hashizume, H., Kitagawa, M., Nishimura, K., Kakaya, N., 1992. Characteristics of 1, 3-bis-(2-ethoxycarbonylchromon-5-yloxy)-2-((S)-lysyloxy)propane dihydrochloride (N-556), a prodrug for the oral delivery of sodium cromoglycate in absorption and excretion in rats and rabbits. J. Pharmacobiol. Dyn. 15, 681–686.

Zou, Y., Priebe, W., Soler-Perez, R., 1996. Lyophilized preliposomal formulation of the non-cross-resistant anthracycline annamycin: effect of surfactant on liposome formation, stability and size. Cancer Chemother. Pharmacol. 39, 103–108.