Iranian Journal of Pharmaceutical Research (2007), 6 (4): 231-242
Received: October 2006
Accepted: February 2007

Original Article

Chitosan-Chondroitin Composite Films: Comparison with *In Vitro* Skin Permeation Data of Hydrophilic and Lipophilic Drugs

Vikas Rana[a], Ashok Kumar Tiwary[a*], Subheet Jain[a] and Dhandeep Singh[b]

[a]*Pharmaceutics Division, Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala.* [b]*Pharmaceutical Chemistry Division, Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala.*

Abstract

Preformulation studies on transdermal dosage forms involve liberal use of animal skin for assessing the permeation characteristics of drugs, influence of permeation enhancers, optimizing the formulation variables etc. The restricted availability of animal skin due to concerns regarding prevention of cruelty to animals has generated considerable interest in developing polymeric films for use as skin substitute during *in vitro* permeation experiments. The present investigation aimed at preparing films containing different ratios of chitosan (CH) to chondroitin sulphate (CS) and rigidizing them by dipping in sodium tripolyphosphate (NaTPP) solution. Statistical optimization designs were employed to screen and optimize the active process and formulation variables that significantly influenced the *in vitro* permeation of 5-fluorouracil (5-FU) and indomethacin (INDO), model polar and non-polar drugs, respectively, across these polyelectric composite (PEC) films. CH to CS ratio, concentration of NaTPP and rigidization time was found to significantly influence the *in vitro* permeation of both drugs. The presence of both sulfonate and phosphonate linkages in PEC films rigidized by 2% w/v NaTPP allowed lowest permeation of either drug. However, films rigidized by 2.5% w/v NaTPP retained predominantly phosphonate linkages and were highly permeable to both drugs. The *in vitro* permeation of both drugs across optimized film formulations was not found to be significantly ($p<0.05$) different as compared to that across rat, rabbit and human epidermal sheets. The optimized PEC films have a great potential to be developed as substitute of animal and human cadaver epidermal sheets for preliminary *in vitro* permeation studies.

Keywords: Optimization; Skin substitute; Chitosan; Chondroitin sulphate; PEC films.

Introduction

Transdermal drug delivery systems are designed to deliver drugs to the systemic circulation through skin. *In vitro* evaluation of these systems during early phase frequently requires animal skin samples. These investigations are necessary for assessing the skin permeation of drug molecules and predicting their *in vivo* performance in humans. However, significant differences between human and animal skin due to variation in thickness, nature of stratum corneum, density of hair follicles, sweat glands (1) etc. makes extrapolation of *in vitro* data generated with animal skin to *in vivo* performance in human beings less reliable. In addition, various factors

* Corresponding author:
E-mail: aktiwary2@rediffmail.com

like race, sex, age and anatomical site of skin influence the reproducibility as well as reliability of data obtained with animal skin experiments. Further, inter-species and intra-species (normal vs diseased skin) differences in skin micro constituents influence reliability of the data generated (2). Moreover, restricted availability of animal skin samples and ethico-legal issues associated with the use of human skin makes it imperative to search for their substitute.

To a large extent, this problem can be overcome by using artificial films instead of animal skin. Artificial films possess distinct advantages over biological membranes in terms of controlled composition, ease of preparation and reproducibility of results (3). The problems of long cell growth cycles and microbial contamination in Caco-2 cell monolayers as well as reduced drug permeation due to absence of sink condition in the static parallel artificial membrane permeability assay method have recently generated interest for investigating lipid impregnated porous supports for predicting gastrointestinal drug absorption (4, 5). Therefore, artificial films have a great potential to be developed as substitute of animal and human cadaver skin for assessing drug transport.

Chondroitin sulphate is a water soluble mucopolysaccharide consisting of D-glucuronic acid linked to N-acetyl-D-galactosamide (6). It contains -COOH and $–HSO_3$ functional groups. -COOH group is reported to covalently bind with collagen in the presence of cross-linking agents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 1,6-diaminohexane, dehydrothermal, 1,1-carboxyldiimidazole and diamines (7-11). Chondroitin sulphate can also electrically bind to polycations such as quaternary ammonium cations (e.g. polydimethyl diallyl ammonium chloride), gelatin, chitosan, cisplatin, peptide/proteins, polyvinyl alcohol and cations like calcium chloride (12-16). Mixing solutions of chondroitin sulphate and chitosan is reported to result in spontaneous ionic interaction leading to the formation of coacervates. This phenomenon has been used for preparing microspheres, beads and matrix tablets for modulating the release of drug molecules (13, 17-19). It is important to note that the literature reveals only the use of ammonium carbamate for masking the cationicity of chitosan with a view of mixing chitosan with sodium alginate without forming coacervates (20). However, there is no report on chitosan-chondroitin sulphate admixtures for preparing clear solutions that could be further dried to form films for controlling drug release.

In the light of these reports, the present investigation aimed at preparing films using chitosan (CH) and chondroitin sulphate (CS) rigidized with sodium tripolyphosphate (NaTPP). Statistical optimization techniques were used for identifying process and formulation variables that were capable of significantly influencing the permeation of 5-fluorouracil (5-FU) and indomethacin (INDO), model polar and non polar drugs, respectively, across these polyelectrolytic composite (PEC) films. Further, attempts were made to optimize the active variables so that the *in vitro* permeation of both drugs across PEC films was not significantly ($p<0.05$) different than that across rat/rabbit/human epidermal sheets.

Experimental

Materials

Chitosan, 95% deacetylation (Indian Sea Foods, Cochin, India), 5-fluorouracil (Dabur Research Foundation, Delhi, India), indomethacin (Crystal Pharmaceuticals, Ambala, India), chondroitin sulphate-A (Panacea Biotech Ltd., Lalru, India) were gift samples and used as received. Sodium tripolyphosphate (NaTPP) and glacial acetic acid were purchased from Loba Chemie, Bombay, India. Other chemical purchased were of analytical or HPLC grade as required.

Methods

Preparation of epidermal sheets

Albino wistar rats (200-350 g) or New Zealand white rabbits (1.5-2.2 kg) of either sex were sacrificed for obtaining their epidermis. Excess chloroform was administered by inhalation to sacrifice the animal and full thickness dorsal skin was excised after removing hairs with electrical hair clipper. The protocol for this study was approved by the institutional animal ethical committee of the Department of

Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India. Human cadaver skin (male) from the abdominal/chest portion was obtained post-mortem within 6 h of death from the Government Medical College and Hospital, Patiala, India after obtaining consent of relatives of the deceased. Epidermal sheets were obtained by soaking the excised whole skin of animal/ humans in phosphate buffer saline, pH 7.4 (PBS) containing trypsin (0.1% w/v) at 60°C for 2 min. Freshly prepared epidermal sheets were mounted on indigenously fabricated vertical Franz diffusion cell apparatus and the receptor fluid (phosphate buffer, pH 7.4) was stirred for 4 h in order to condition the epidermal sheet before commencing *in vitro* permeation experiments.

Chitosan-chondroitin sulphate-sodium tripolyphosphate PEC films

Formulation design

The process and formulation variables involved in the preparation of PEC films were screened for their effect on permeation of 5-FU and INDO. Plackett-Burman screening design (PBD) was used to formulate PEC films for screening the effect of process and formulation variables on permeation parameters of 5-FU and INDO. Various films were prepared by using low and high levels (designated -1 and +1, respectively) of each variable (X1-X8). The permeation of both 5-FU and INDO across these film formulations (P1-P8) summarized in Table 1 was studied using vertical Franz diffusion cells. The active variables (that significantly influenced permeation of either drug) were used for formulating additional films (Table 2) according to central composite design (CCD). CCD was employed in order to investigate the influence of active variables [ratio of CH: CS (X1), concentration of NaTPP (X2), rigidization time (X3)] on permeation of both drugs in the entire experimental domain. Permeation of both drugs across these films was compared with that obtained across rat/rabbit/human epidermal sheets. Multiple linear regression was performed by employing statistica software-7.0 (Sta Soft Inc., Tulsa, USA) for evaluating the influence of active variables obtained from CCD on the *in vitro* flux of both drugs.

Preparation of films

The total polymer content in all the films was 4% w/v. Solution of CH (60-40% w/w of total polymer) was prepared in 30 ml of acetic acid (3% v/v) with simultaneous homogenization (2000 rpm) and addition of 5 ml of ammonium acetate (14-17 M). CS (40-60% w/w of total polymer) was dissolved separately in water (10 ml) and added drop wise to chitosan solution with constant stirring. The volume was adjusted to 60 ml with water. The clear mixture was degassed under vaccum (10 psig), poured in petridish and dried at 60 °C for 24-36 h. The dried films were stored in polyethylene bags till use. These CH-CS films were rigidized by dipping in 20 ml of NaTPP solution (0.5-2.5% w/v) adjusted to pH 5.0 for 15-45 min. After treatment the resultant PEC films were washed with water to remove excess NaTPP, dried at 60 °C for 8 h and stored till further use. The PEC films were mounted on Franz diffusion cells and the receptor fluid (phosphate buffer, pH 7.4) was stirred for 4h in order to condition the films before commencing *in vitro* permeation studies.

Physicochemical characterization of films

Preparation of PEC films

Films used for physicochemical characterization were prepared by employing CH:CS ratio of 60:40 according to the procedure described above. These films were rigidized by dipping in 20 ml of NaTPP solution (0.5, 1.0, 1.5, 2.0 or 2.5% w/v, adjusted to pH 5.0 with HCl) for 45 min to obtain different PEC films. Films were dried at 60°C for 8 h and stored in air tight polyethylene bags till further use.

Differential scanning calorimetric (DSC) analysis

Samples of chitosan powder, chondroitin sulphate powder or PEC films dried to constant weight were subjected to DSC analysis (Mettler Toledo Star System, 821E, Switzerland) employing a heating rate of 10 °C/min. Each experiment was carried out in triplicate.

Infrared absorption spectroscopy (IR)

Dried PEC films were triturated with an equal quantity of KBr and compressed to obtain discs for IR analysis. The spectra of these discs

Table 1. Plackett Burman design for identifying active formulation and process variables influencing flux of indomethacin (INDO) and 5-fluorouracil (5-FU) across PEC films.

Batch no.	X1 (%, w/w)	X2 (%, w/v)	X3 (min)	X4 (M)	X5 (h)	X6	X7	INDO flux[a] ($\mu g\ h^{-1}\ cm^{-2}$)	5-FU flux[a] ($\mu g\ h^{-1}\ cm^{-2}$) X 10^3
P1	+1(60:40)	+1(2)	+1(45)	-1(13)	1(48)	-1	-1	25.365±2.4	0.523±0.046
P2	-1(40:60)	+1(2)	+1(45)	+1(20)	-1(24)	+1	-1	222.615±5.6	4.590±0.045
P3	-1(40:60)	-1(1)	+1(45)	+1(20)	+(48)	-1	+1	288.320±3.6	5.920±0.010
P4	+1(60:40)	-1(1)	-1(15)	+1(20)	+1(48)	+1	-1	127.550±0.2	2.630±0.003
P5	-1(40:60)	+1(2)	-1(15)	-1(13)	+1(48)	+1	+1	253.170±0.2	5.220±0.008
P6	+1(60:40)	-1(1)	+1(45)	-1(13)	-1(24)	+1	+1	93.265±2.7	1.923±0.014
P7	+1(60:40)	+1(2)	-1(15)	+1(20)	-1(24)	-1	+1	59.170±0.5	1.220±0.002
P8	-1(40:60)	-1(1)	-1(15)	-1(13)	-1(24)	-1	-1	321.215±5.1	6.623±0.097

X1- Ratio of CH to CS; X2- concentration of NaTPP; X3- rigidization time; X4- concentration of ammonium acetate; X5- drying time at 60°C; X6 , X7- dummy variables; +1 and -1 are transformed values of real experimental values shown in parentheses.
[a] values represent mean ± SD of 5 experiments.

were recorded on a Perkin Elmer RXI, IR spectrophotometer (USA) in the spectral region of 500 to 4000 cm^{-1}. Each spectrum was recorded in triplicate.

Atomic absorption spectroscopy for Na^+ in PEC films

Dried PEC films were digested in 2 ml of aqua regia and evaporated to dryness on a water bath. The residue was cooled, dissolved in 10 ml of HCl (50% v/v) and filtered through a G_3 filter. The filtrate obtained was subjected to flame atomic absorption spectroscopy for estimation of Na^+ (GBC, 932 AAS, and Australia). All estimations were performed in triplicate.

Table 2. Central composite design using active formulation and process variables influencing flux of 5-fluorouracil (5-FU) and indomethacin (INDO) across PEC films.

Batch no.	X1 (%, w/v)	X2 (%, w/v)	X3 (min)	5-FU flux[a] ($\mu g\ h^{-1}\ cm^{-2}$) X 10^3	INDO flux[a] ($\mu g\ h^{-1}\ cm^{-2}$)
1F	-1(40:60)	-1(1)	-1(15)	6.623±0.097	321.215±5.1
2F	+1(60:40)	-1(1)	-1(15)	2.630±0.002	127.550±0.2
3F	-1(40:60)	+1(2)	-1(15)	5.220±0.008	253.170±0.2
4F	+1(60:40)	+1(2)	-1(15)	1.220±0.002	59.170±0.5
5F	-1(40:60)	-1(1)	+1(45)	5.920±0.010	288.320±3.6
6F	+1(60:40)	-1(1)	+1(45)	1.923±0.014	93.265±2.7
7F	-1(40:60)	+1(2)	+1(45)	4.590±0.045	222.615±5.5
8F	+1(60:40)	+1(2)	+1(45)	0.523±0.046	25.365±1.2
1S	-1.682(33:67)	0(1.5)	0(30)	1.234±0.050	59.849±2.3
2S	+1.682(67:33)	0(1.5)	0(30)	5.123±0.056	247.440±0.7
3S	0(50:50)	-1.682(0.659)	0(30)	4.563±0.023	221.315±0.6
4S	0(50:50)	+1.682(2.34)	0(30)	2.632±0.090	127.125±4.9
5S	0(50:50)	0(1.5)	-1.682(5)	4.237±0.069	204.647±7.3
6S	0(50:50)	0(1.5)	+1.682(55.2)	2.932±0.044	141.908±1.1
1C	0(50:50)	0(1.5)	0(30)	3.323±0.006	161.165±1.4
2C	0(50:50)	0(1.5)	0(30)	3.583±0.012	173.058±1.3
3C	0(50:50)	0(1.5)	0(30)	3.452±0.009	166.131±1.2
4C	0(50:50)	0(1.5)	0(30)	3.632±0.012	175.425±1.4

X1- Ratio of CH to CS; X2- concentration of NaTPP; X3- rigidization time; F- factorial design, S- star design, C- centre points.
[a] values represent mean ± SD of 5 experiments.

In vitro permeation studies

Vertical Franz diffusion cell apparatus was designed and fabricated in our laboratory. It consisted of 8 glass diffusion cells (20 ml each) maintained at 37°C±1°C by water heating system. Stirring of receptor fluid in each cell was accomplished by magnetic stirrers (300 rpm). PEC films or epidermal sheets were clamped between donor and receptor compartments. The receptor compartment contained phosphate buffer (pH 7.4), sodium azide (0.5% w/v) as preservative and PEG 400 (5.0% v/v) for maintaining sink condition. Either drug was suspended in propylene glycol (4 ml) and loaded in the donor compartment. Aliquots (1 ml) withdrawn at various intervals were immediately analyzed for 5-FU or INDO by HPLC (Waters, 515 pump, USA) using Spherisorb C_{18} column (4.6 x 250 mm) and UV detector (2487 Dual wavelength). Sodium acetate (0.1% w/v) or methanol: citrate buffer 10 $mmol^{-1}$ (75:25) at flow rates of 0.6 ml min^{-1} or 1.0 ml min^{-1}, respectively, was used as mobile phase for 5-FU or INDO. The respective detection wavelength for 5-FU or INDO was 265 nm or 240 nm as reported by Sasaki *et al*. (21). Cartesian plots of cumulative amount of 5-FU or INDO permeated into receptor compartment versus time were plotted. Flux ($\mu g/h/cm^2$) was calculated from the slope of steady state linear portion of the plot. All permeation experiments were replicated five times.

Results and Discussion

The thickness of films prepared using different ratios of CH: CS was 0.16-0.32 mm. For comparison, the thickness of epidermal sheets obtained from rat/rabbit/humans was 0.17-0.29 mm. The total polymer concentration was deliberately fixed at 4% w/v so that polymeric films could be prepared with thickness comparable to epidermal sheets in order to negate the influence of thickness on drug permeation.

Results obtained using films prepared according to PBD revealed that the ratio of CH to CS (X1), concentration of NaTPP solution (X2) used for rigidizing films and rigidization time (X3) significantly ($p<0.05$) influenced the flux of both 5-FU (model polar drug) and INDO (model non polar drug). The data is summarized in Table 1. The effect of various formulation and process variables (X1…X7) on 5-FU flux (Y1) across these PEC films was found to be represented by the equation Y1=3.58-2.00X1-0.693X2- 0.342X3+0.01X4 - 0.01X5+ 0.009X6-0.01X7 and that for INDO flux (Y2) by the equation Y2=173.830-97.490X1-33.753X2 -16.440X3+0.580X4-0.232X5+0.316X6-0.352X7. These three factors (X1, X2 and X3) that were found active from the PBD were evaluated over a larger experimental domain by preparing additional film formulations (Table 2) using CCD.

Multiple linear regression of the data obtained from CCD indicated that all the active variables significantly influenced ($p<0.05$) the permeation of both drugs. The effect of various active formulation and process variables (X1, X2, X3) including their interection effects (X1X2, X1X3, X2X3 and X1X2X3) on 5-FU flux (Y1) across these PEC films was found to be represented by the equation Y1=3.52-0.696X1-0.643X2-0.361X3-0.009X1X2-0.008X1X3+0.010X2X3 -0.007X1X2X3 and that for INDO flux (Y2) by equation Y2=170.48-34.004 X1-31.36X2-17.35X3-0.316X1X2-0.58X1X3+0.352X2X3 -0.232X1X2X3. It was evident from these equations that the magnitude of interaction terms was significantly less ($p<0.05$) as compared to that of main terms. This indicated that the interaction terms did not play any significant role in influencing the permeation of both drugs across PEC films. Therefore, these interaction terms were not considered subsequently while analyzing the influence of active variables on drug permeation.

Figure 1 depicts the response surface plots representing the influence of active variables (X1, X2 and X3) on 5-FU flux (Y1). Figure 2 shows the response surface plots for the influence of active variables (X1, X2 and X3) on INDO flux (Y2). The permeation of 5-FU (Y1) or INDO (Y2) was observed to decrease linearly with an increase in CH:CS ratio (X1) as well as increase in concentration (X2) of NaTPP (Figures 1A and 2A). The more or less flat nature of these response plots suggested almost similar influence of X1 and X2 in decreasing the flux of both drugs. Further, evaluation of the response plots revealed approximately 1.9-fold pronounced

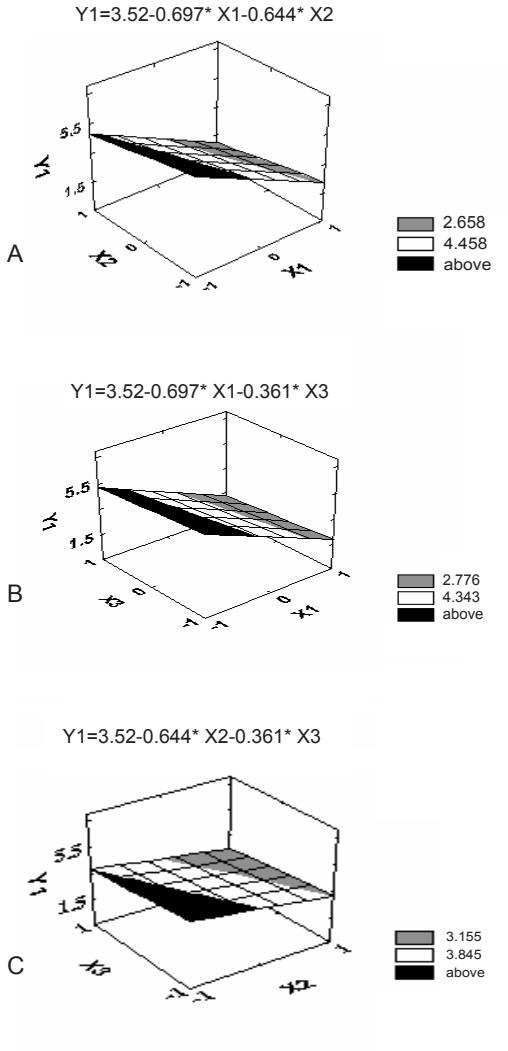

Figure 1. Response surface plots depicting the influence of: A, Ratio of CH:CS (X1) and concentration of NaTPP (X2); B, Ratio of CH:CS (X1) and Rigidization time (X3); C, Concentration of NaTPP (X2) and Rigidization time (X3) on 5-FU flux (Y1).

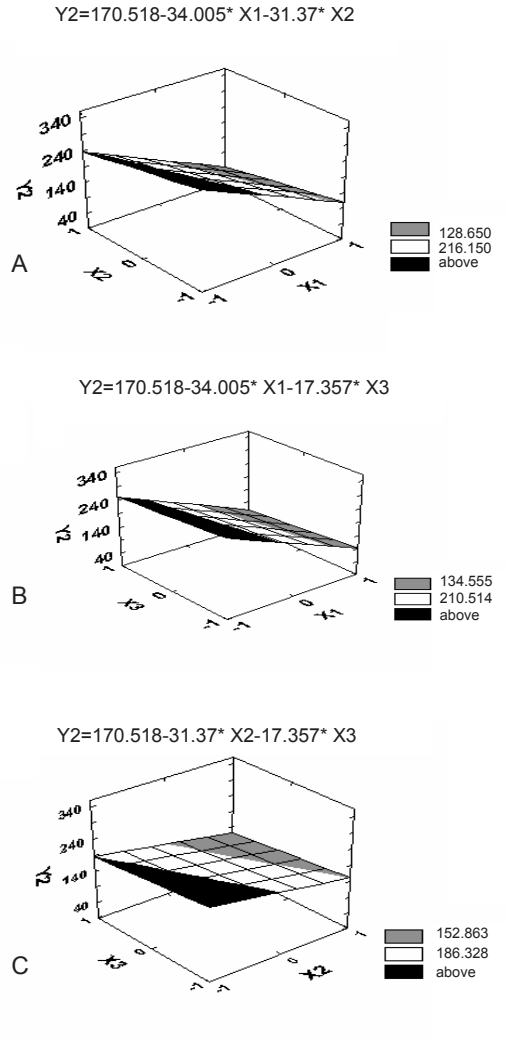

Figure 2. Response surface plots depicting the influence of: A, Ratio of CH:CS (X1) and concentration of NaTPP (X2); B, Ratio of CH:CS (X1) and Rigidization time (X3); C, Concentration of NaTPP (X2) and Rigidization time (X3) on INDO (Y2).

influence of CH:CS ratios (X1) as compared to that of rigidization time (X3) in reducing the permeation of both drugs (Figures 1B and 2B). Similarly, an augmentation of X2 levels was observed to produce an approximately 1.8-fold greater reduction in permeation of both drugs as compared to that produced by an increase in X3 levels (Figure 1C and 2C). It is important to note that all the NaTPP solutions used for rigidizing CH-CS films were adjusted to pH 5.0. This was done with an aim to favour interaction between CH and CS as well as to cross-link CH (that had not reacted with CS) with NaTPP (22). This treatement rendered these PEC films insoluble in phosphate buffer (pH 7.4), thus ensuring their integrity during *in vitro* permeation studies. The flux of both 5-FU and INDO was observed to decrease linearly across films rigidized using 0.5-2% w/v concentration of NaTPP. However, the flux of both drugs increased significantly ($p<0.05$) across films that were rigidized by dipping in higher concentration (2.5% w/v) of NaTPP solution (Table 3).

The DSC thermograms of PEC films prepared from 60:40 mixture of CH:CS and rigidized by dipping in various concentrations of NaTPP solution are depicted in Figure 3. The physicochemical characteristics of these

Table 3. Permeation of INDO and 5-FU across films prepared with different ratio of CH and CS followed by rigidization by dipping in NaTPP solution (pH 5.0).

NaTPP concentration (%, w/v)	5-FU flux[a] (μg h^{-1} cm^{-2}) X 10^3		INDO flux[a] (μg h^{-1} cm^{-2})	
	CH:CS ratio		CH:CS ratio	
	60:40	40:60	60:40	40:60
0.5	2.635±0.050	6.630±0.093	127.797±7.8	321.555±2.4
1.0	1.923±0.014	5.920±0.010	93.265±2.7	287.120±3.6
1.5	1.230±0.050	5.340±0.023	59.655±2.4	258.990±5.8
2.0	0.523±0.046	4.590±0.045	25.365±2.4	222.615±5.6
2.5	6.693±0.023	11.732±0.062	324.61±6.7	569.002±3.2

[a] values represent mean±SD of 5 experiments.

films are summarized in Table 4. It is evident from Figure 3A that CH powder exhibited one endothermic and exothermic transition each at, respectively, 70.2 °C and 311.30 °C. The films prepared by dissolving CH alone in acetic acid (3.0% v/v) exhibited similar thermograms with one endothermic and exothermic transition but at different temperatures (Figure 3B) as compared to CH powder. The DSC thermogram of CS revealed one endotherm at 117 °C and one exotherm at 239.69 °C (Figure 3C). However, films prepared by mixing 60:40 ratio of CH (in 3.0% v/v acetic acid) in the presence of 5 ml of 14 M ammonium acetate with an aqueous solution of CS exhibited a large endotherm at 129.04 °C followed by another endotherm at 189.22 °C (Figure 3E). The enthalpy (ΔH) associated with large endotherm seems to be an approximate summation of the ΔH associated with CH (Figure 3B) and CS (Figure 3C) films. It is noteworthy that the reaction between CH and CS was prevented by the addition of high concentration of ammonium acetate. Hence, the first large endothermic transition in CH-CS films (Figure 3E) could be ascribed to the presence of a physical mixture of chitosan acetate and CS. The second endothermic transition at 189 °C should have arisen due to interaction between these polymers and would have occurred due to evaporation of ammonia and acetic acid during drying of films. Therefore, this endothermic peak (189 °C) can be assigned to the interaction between CH and CS.

The thermograms of PEC films obtained after rigidizing CH-CS films by dipping in various concentrations of NaTPP solution (0.5-2.5% w/v, pH 5.0) revealed two endothermic transitions in addition to a large endotherm (Table 4). A comparison of the thermograms of these PEC films (Figures 3F-J) revealed that the ΔH of the first endothermic transition

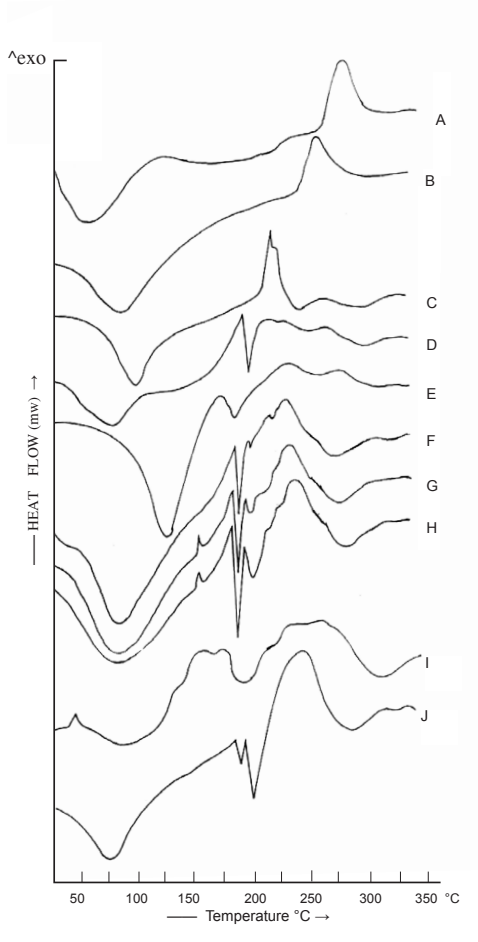

Figure 3. DSC thermograms of: A, chitosan powder; B, chitosan acetate films; C, chondroitin sulphate powder; D, chitosan (4%, w/v) films rigidized with NaTPP (1% w/v); E, CS:CH (60:40) films and CS:CH (60:40) films rigidized by dipping in NaTPP (% w/v, pH 5.0) solution (PEC films) F, 0.5; G, 1.0; H, 1.5; I, 2.0 and J, 2.5.

Table 4. Physicochemical characteristics of PEC films prepared with 60:40 ratio of CH:CS and rigidized with different concentrations of NaTPP solution (pH 5.0).

NaTPP concentration (%, w/v)	Na^+ (µg per mg of film)[a]	DSC analysis[a]					
		FIRST ENDOTHERM		SECOND ENDOTHERM		THIRD ENDOTHERM	
		T_m (°C)	ΔH (J g^{-1})	T_m (°C)	ΔH (J g^{-1})	T_m (°C)	ΔH (J g^{-1})
0.5	15.64±0.2	96.20±1.2	238.83±1.2	192.23±1.2	21.32±1.2	213.23±1.3	15.32±1.5
1.0	17.87±0.3	93.20±1.4	232.85±2.3	192.33±1.2	21.97±2.3	208.89±1.8	24.47±1.1
1.5	19.83±0.21	95.33±1.1	228.32±3.2	190.34±1.1	23.32±3.2	209.23±1.5	30.87±2.1
2.0	21.45±0.23	126.01±1.5	220.58±3.3	200.33±1.5	26.18±1.3	*	*
2.5	24.86±0.10	95.10±1.2	82.36±1.2	192.27±1.5	4.76±3.3	208.14±1.4	36.33±2.5

[a] values represent mean±SD of 3 experiments; *not observed.

decreased with an increase in the concentration of NaTPP used for rigidization. The appearance of first endothermic transition was assigned to the presence of appreciable quantities of CH and CS in non rigidized CH-CS films (Figure 3E). Hence, the observed reduction in ΔH of the first endotherm could be possibly due to interaction of greater quantity of CH with CS and/or CH with NaTPP during rigidization process which resulted in reduced content of both polymers in the physical mixture. However, ΔH of second endothermic transition remained approximately the same (Figure 3F-H) in PEC films rigidized by dipping in 0.5%-1.5% w/v NaTPP solution (Table 4). This can be ascribed to the fact that all NaTPP solutions were adjusted to pH 5.0, which ensured equal magnitude of pH-dependent interaction between CH and CS (22). The third endothermic transition in PEC films (Figure 3F-J) appeared between 208 °C-213 °C. It is noteworthy that this endothermic peak appeared at the same temperature as the endothermic transition obtained in films prepared with CH alone and rigidized with NaTPP (Figure 3D). Hence, appearance of the third endothermic transition in PEC films can be suggested to indicate the phosphonate linkage between $-NH_3^+$ groups of CH (that had not reacted with CS) and $-PO_3^-$ groups of NaTPP. The ΔH of this third endotherm increased till the concentration of NaTPP used for rigidization increased to 1.5% w/v (Table 4). Figure 3I revealed merger of second and third endothermic transitions when 2.0% w/v NaTPP was used for rigidizing films. This indicates maximum interaction between CH and CS as well as between CH and NaTPP at 2% w/v concentration of NaTPP solution. However, further increase in concentration of NaTPP to 2.5% w/v probably resulted in weakening of CH-CS interaction (decreased ΔH of second endotherm) and marked strengthening (increased ΔH of third endotherm) of CH-NaTPP interaction (Figure 3J).

The Na^+ content in PEC films was found to increase with an increase in the concentration of NaTPP used for rigidization. This indicated enhanced interaction between CH and NaTPP with increasing concentration of NaTPP. The highest Na^+ content was found in films rigidized with 2.5% w/v NaTPP solution. These observations are consistent with results of DSC analysis, where the ΔH of the third endothermic transition was found to invariably increase with an increase in NaTPP concentration during rigidization (Table 4).

The IR spectra of chitosan acetate showed peaks at 1560 cm^{-1} and 1412 cm^{-1} indicating presence of ammonium ion and carboxylate ion, respectively (Figure 4A). CS powder gave peaks at 1646 cm^{-1}, 1416 cm^{-1} and 1130 cm^{-1} corresponding to $-CONH_2$, $-COO^-$ and $-HSO_3^-$ groups,[23] respectively (Figure 4B). The films prepared by mixing CH and CS in presence of ammonium acetate (5 ml of 14 M) gave a very small peak at 1155 cm^{-1}. The characteristic peak of $NH_3^+SO_3^-$ is reported to occur in the range of 1150-1300 cm^{-1} (22). Hence, the occurrence of the weak peak at 1155 cm^{-1} indicated the presence of sulphonate linkages between $-NH_3^+$ of CH and $-HSO_3^-$ of CS (Figure 4D). Rigidization of CH-CS films by dipping in 0.5-1.5% w/v NaTPP solutions made the peak at 1155 cm^{-1} more prominent indicating significant sulphonate linkages. In addition,

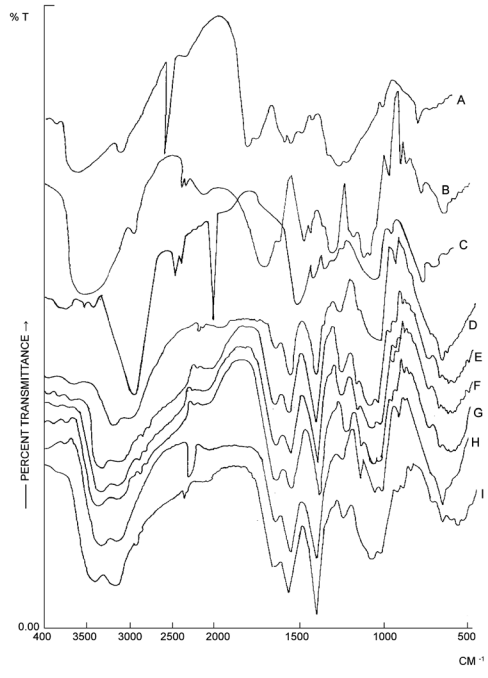

Figure 4. IR spectra of: A, chitosan acetate film; B, chondroitin sulphate powder; C, chitosan (4% w/v) films rigidized with NaTPP (1% w/v); D, CS:CH (60:40) films and CS:CH (60:40) films rigidized by dipping in NaTPP (% w/v, pH 5.0) solution (PEC films) E, 0.5; F, 1.0; G, 1.5; H, 2.0 and I, 2.5.

appearance of a peak at 1076 cm^{-1} suggested the formation of phosphonate linkages between $-NH_3^+$ groups of CH (that had not reacted with CS) and $-PO_3^-$ groups of NaTPP (Figure 4E-G). A similar peak at 1072 cm^{-1} was observed to occur in films prepared using CH alone after rigidization with NaTPP (Figure 4C). Films rigidized with 2% w/v NaTPP solution produced intense peaks both at 1155 cm^{-1} and 1076 cm^{-1} suggesting strong sulphonate and phosphonate linkages, respectively (Figure 4H). However, dipping in solutions containing higher (2.5% w/v) concentration of NaTPP resulted in abolition of the peak at 1155 cm^{-1} and broadening of the peak at 1078 cm^{-1}. This indicated a decrease in sulphonate linkages while retaining the phosphonate linkages (Figure 4I). These findings are supportive of the DSC results where the ΔH of second endothermic transition (characteristic of CS-CH interaction) was observed to decrease 6-fold in films rigidized by 2.5% w/v NaTPP solution. This was accompanied with an increase in the ΔH of third endothermic transition (characteristic of CH-NaTPP interaction). Hence, it seems probable that rigidization of films by dipping in 2.5% w/v NaTPP solution weakened the sulphonate linkages between $-HSO_3^-$ of CS and $-NH_4^+$ of CH as well as strengthened the phosphonate linkages between $-PO_3^-$ of NaTPP and $-NH_4^+$ of CH.

It is evident from Table 3 that the permeation of both drugs across PEC films decreased with the use of increasing concentrations of NaTPP during rigidization. The flux of both drugs was minimum across PEC films rigidized by dipping in 2.0% w/v NaTPP solution. The results of both DSC analysis (Figure 3I) and IR spectroscopy (Figure 4H) revealed considerable interaction between CH and CS as well as between CH and NaTPP in this PEC film. On the other hand, films rigidized with lower concentrations of NaTPP exhibited predominant presence of CH-CS interaction with negligible contribution of CH-NaTPP interaction. Hence, the presence of both interactions could be suggested to restrict the permeation of 5-FU and INDO across PEC films rigidized by 2.0% w/v NaTPP. However, after rigidization of films with 2.5% w/v NaTPP, the results of both DSC analysis (Figure 3J) and IR spectroscopy (Figure 4I) showed decreased sulphonate linkages accompanied with enhanced phosphonate linkages. Therefore, it can be postulated that reduction in sulphonate linkages perhaps played a critical role and the resultant films could not prevent high permeation of both drugs across PEC films rigidized by 2.5% w/v NaTPP.

The three equations generated by using statistica software (version 7.0) for both drugs (Table 5) were solved for calculating optimum values of X1 (ratio of CH:CS), X2 (concentration of NaTPP) and X3 (rigidization time) for preparing PEC films that would exhibit flux comparable to that across animal/human epidermal sheets. The lower and upper solved values of all the active variables (X1, X2, X3) summarized in Table 5 were employed to prepare films in order to simulate the *in vitro* flux of 5-FU or INDO across rat, rabbit and human cadaver epidermal sheets. Table 6 shows that the permeation of both 5-FU and INDO across PEC films formulated by using various optimized values of active variables did not differ significantly ($p<0.05$) as compared to that across rat/rabbit/human epidermal sheet. Figure

Table 5. Equations for relating X1 (ratio of CH to CS), X2 (concentration of NaTPP) and X3 (rigidizing time) with flux of 5-FU (Y1) and INDO (Y2) across PEC films.

Treatment	Equation	
X1 *vs* X2	Y1=3.52-0.697X1-0.644X2	Y2 =170.52- 34.00X1-31.40X2
X2 *vs* X3	Y1=3.52-0.697X2-0.361X3	Y2 =170.52-34.00X2-17.36X3
X3 *vs* X1	Y1=3.52-0.644X1-0.361X3	Y2 =170.52-31.40X1-17.36X3

	Solved Value and Optimized Film Composition					
	Optimized Variable For Films Simulating Flux of					
	5-FU			INDO		
Epidermis Type	X1 (% w/w)	X2 (% w/v)	X3 (min)	X1 (% w/v)	X2 (% w/v)	X3 (min)
RAT	59.5:40.5 - 61.6:38.4	2.01-2.13	58-64	62:38 - 61.3:38.7	2.14-2.11	64.8-62.5
RABBIT	58:42 - 57:43	1.92-1.90	53-51	44.1:55.9 - 41.82:58.18	1.18-1.07	13-7
HUMAN	70.3:29.7 - 69.8:30.2	2.6	89-87	71:29 - 69:31	2.61-2.57	91-88

5A and 5B represent the insignificant difference in permeation of 5-FU and INDO across optimized PEC films with respect to epidermal membranes.

Figure 6 and Figure 7 show a high correlation for the permeation of 5-FU and INDO, respectively, across optimized PEC films and that across animal/human epidermal sheets. This suggests that these optimized PEC films can be used for simulating the permeation of polar and non polar drugs across rat/rabbit/human epidermis. However, the optimized values for preparing PEC films capable of simulating flux of either drug are different for epidermal

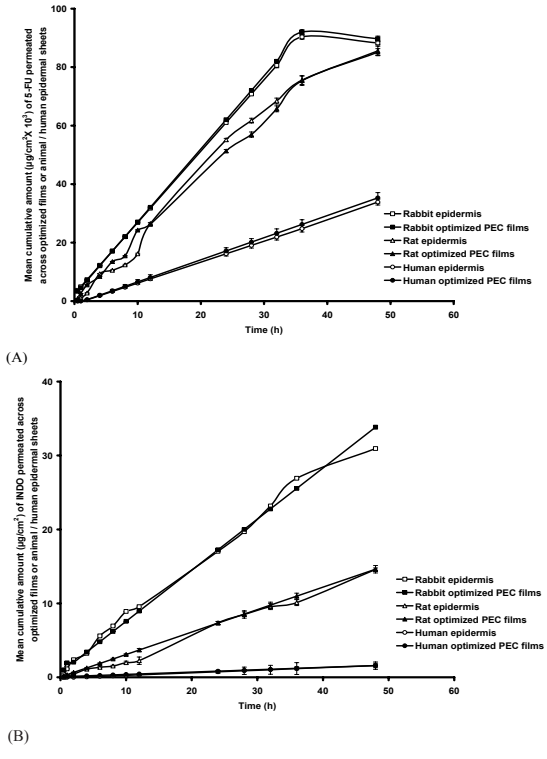

Figure 5. Comparison of permeation of 5-FU (A) and INDO (B) across epidermal membranes wit respect to optimized PEC films.

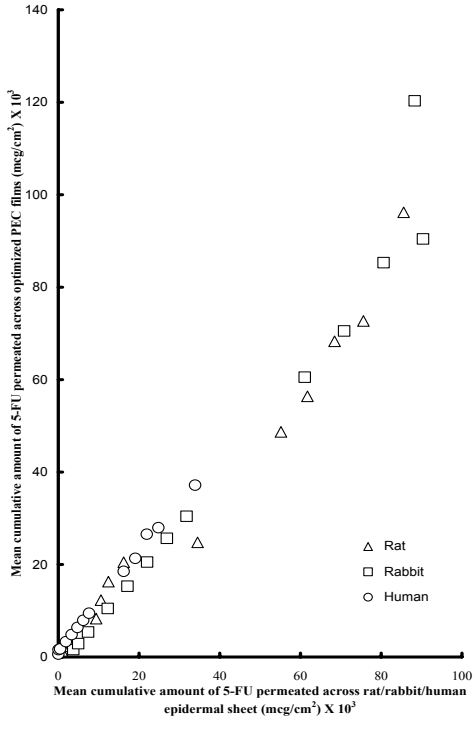

Figure 6. Correlation between *in vitro* permeation of 5-FU across optimized PEC films and rat epidermal sheet (Y=0.988 X+0.253, R^2=0.97), rabbit epidermal sheet (Y =1.155 X-4.470, R^2 =0.96) and human cadaver epidermal sheet (Y=1.0804 X+1.251, R^2=0.99). Each value represents mean±SD, n=5.

Table 6. Comparison of permeation of 5-FU or INDO across optimized PEC films with that across excised rat/rabbit/human epidermal sheets.

Animal Epidermis		Optimized Films	Statistical Difference
Type	Flux[a] (Epidermal sheet)	Flux[a]	
5-Fluorouracil	($\mu g\ h^{-1}\ cm^{-2}$) X 10^3		
Rat	2.05±0.15	2.0±0.56	NS
Rabbit	2.47±0.03	2.5±0.32	NS
Human	0.72±0.03	0.77±0.13	NS
Indomethacin	($\mu g\ h^{-1}\ cm^{-2}$)		
Rat	92.6±2.5	96.43±0.36	NS
Rabbit	217.6±7.5	227.32±0.66	NS
Human	34.2±3.2	38.23±0.32	NS

Comparison between epidermal sheets and optimized PEC films ($p<0.05$): NS - no significant difference, [a] values represent mean±SD of 5 experiments.

sheets of different animals. This could be due to the different inherent permeabilities of these epidermal sheets due to variation in their biochemical constituents, anatomical ultrastructure etc. Nevertheless, the findings strongly indicate a great potential of CH-CS films for use as skin substitute during the preliminary *in vitro* permeation investigations on transdermal formulations.

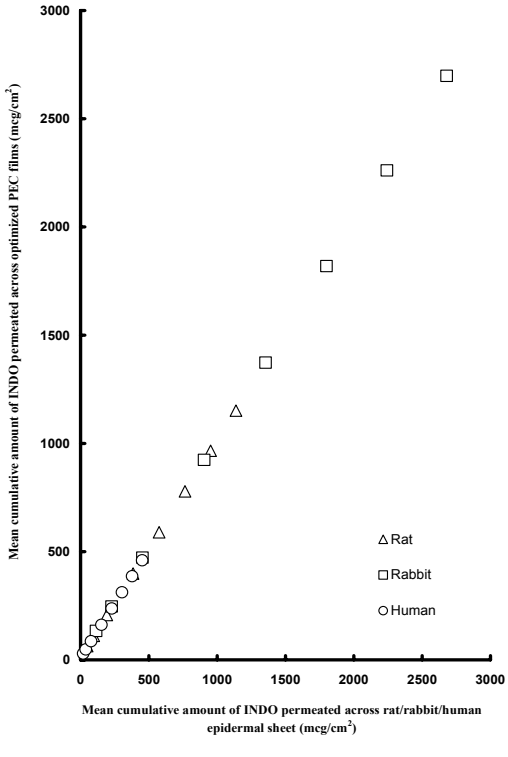

Figure 7. Correlation between *in vitro* permeation of INDO across optimized PEC films and rat epidermal sheet (Y=099 X+15.05, R^2=1.00), rabbit epidermal sheet (Y=0.99 X+ 19.95, R^2=1.00) and human cadaver epidermal sheet (Y=0.99 X+10.089, R^2=1.00). Each value represents mean±SD, n=5.

Conclusion

In conclusion, the results of these investigations suggest that the *in vitro* permeation of both 5-FU (model polar drug) and INDO (model non polar drug) across animal/human epidermal sheets could be simulated by using optimized CH-CS films after rigidization with NaTPP solution. The permeation of drugs across these PEC films was found to be modulated by the nature of interaction between CH, CS and NaTPP. In addition, the concentration of NaTPP used for rigidization these films was found to play a critical role in influencing the permeation of both drugs. This knowledge of interaction between CH and CS and between CH and NaTPP after rigidization with different concentrations of NaTPP has a great potential not only for developing PEC films as substitutes of animal/human epidermis but also for modulating release of drugs from other dosage forms. Although, the results of this investigation confirmed the suitability of optimized CH-CS PEC films as substitute of animal/human epidermal sheets for 5-FU and INDO, additional work on drugs with diverse physicochemical properties is advocated to

confirm the universal validity of these films for use during preformulation studies on transdermal formulations.

Acknowledgment

We would like to express our sincere thanks to Indian Sea Foods, Cochin, India, Panacea Biotech Ltd, Lalru, India, Dabur Research Foundation, Delhi, India and Crystal Pharmaceutical, Ambala, India for providing gift samples of chitosan, chondroitin sulphate-A, 5-fluorouracil and indomethacin, respectively.

References

(1) Barry BW. Novel mechanism and devices to enable successful transdermal drug delivery. *Eur. J. Pharm. Sci*. (2001) 14: 101-114

(2) Nardo AD, Wertz P, Giannetti A and Seidenari S. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm. Venereol.* (1985) 78: 27-30

(3) Feldstein MM, Raigorodskii IM, Iordanskii AL and Hadgraft J. Modeling of percutaneous drug transport in vitro using skin-immitating carbosil membrane. *J. Control. Rel*. (1998) 52: 25-40

(4) Corti G, Maestrelli F, Cirri M, Furlanetto S and Mura P. Development and evaluation of an *in vitro* method for prediction of human drug absorption I. Assessment of artificial membrane composition. *Eur. J. Pharm. Sci*. (2006) 27: 346-353

(5) Corti G, Maestrelli F, Cirri M, Zerrouk N and Mura P. Development and evaluation of an in vitro method for prediction of human drug absorption II. Demonstration of method suitability. *Eur. J. Pharm. Sci*. (2006) 27: 354-362

(6) Toledo OMS and Dietrich CP. Tissue specific distribution of sulphated mucopolysaccharides in mammals. *Biochem. Biophys. Acta* (1977) 498: 114-122

(7) Kataropoulou M, Henderson C and Grant H. The influence of glycosaminoglycans and crosslinking agents on the phenotype of hepatocytes cultured on collagen gels. *Hum. Exp. Toxicol.* (2003) 22: 65-71

(8) Pieper JS, Oosterhof A, Dijkstra PJ, Veerkamp JH and Van Kuppevelt TH. Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. *Biomaterials* (1999) 20: 847-858

(9) Osborne CS, Barbenel JC, Smith D, Savakis M and Grant MH. Investigation into the tensile properties of collagen/chondroitin-6-sulphate gels: the effect of crosslinking agents and diamines. *Med. Biol. Eng. Comput.* (1998) 36: 129-134

(10) Hanthamrongwit M, Reid WH and Grant MH. Chondroitin-6-sulphate incorporated into collagen gels for the growth of human keratinocytes: the effect of cross-linking agent and diamines. *Biomaterials* (1996) 17: 775-780

(11) Sintov A, Di-Capua N and Rubinstein A. Cross-linked chondroitin sulphate: characterization for drug delivery purposes. *Biomaterials* (1995) 16: 473-485

(12) Lee C, Kung P and Lee Y. Preparation of poly (vinyl alcohol)-Chondroitin sulphate hydrogel as matrices in tissue engineering. *Carboh. Polymers* (2005) 61: 348-354

(13) Kofuji K, Ito T, Murata Y and Kawashima S. Effect of chondroitin sulphate on the biodegradation and drug release of chitosan gel beads in subcutaneous air pouches of mice. *Bio. Pharm. Bull.* (2002) 25: 268-271

(14) Zhang JS, Imai T, Suenaga A and Otagiri M. Molecular-weight-dependent pharmacokinetics and cytotoxic properties of cisplatin complexes prepared with chondroitin sulfate A and C. *Int. J. Pharm.* (2002) 240: 23-31

(15) Park YJ, Lee JY, Seol YJ, Chung CP and Lee SJ. Controlled release of platelet-derived growth factor-BB from chondroitin sulfate-chitosan sponge for guided bone regeneration. *J. Contr. Rel.* (2000) 67: 385-394

(16) Kuijpers AJ, Van Wachem PB, Van Lutn MJA, Brouwer LA, Engbers GHM, Krijgsveld J, Zaat SAJ, Dankert J and Feijen J. *In vitro* and *in vivo* evaluation of gelatin-chondroitin sulphate hydrogels for controlled release of antibacterial proteins. *Biomaterials* (2000) 21: 1763-1772

(17) Kofuji K, Ito T, Murata Y and Kawashima S. The controlled release of a drug from biodegradable chitosan gel beads. *Chem. Pharm. Bull.* (2000) 48: 579-581

(18) Ganza-Gonzalez A, Anguiano-Igea S, Otero-Espinar FJ and Mendez JB. Chitosan and chondroitin microspheres for oral-administration controlled release of metaclopramide. *Eur. J. Pharm. Biopharm*. (1999) 48: 149-155

(19) Rubinstein A, Nakar D and Sintov A. Chondroitin sulfate: A potential biodegradable carrier for colon-specific drug delivery. *Int. J. Pharm.* (1992) 84: 141-150

(20) Muzzarelli C, Tosi G, Francescangeli O and Muzzarelli RA. Alkaline chitosan solutions. *Carbohydr. Res.* (2003) 338: 2247-22455

(21) Sasaki H, Kojima M, Mori Y, Nakamura J and Shibasaki J. Enhancing effect of pyrrolidone derivatives on transdermal penetration of 5-fluorouracil, triamcinolone acetonide, indomethacin, and flurbiprofen. *J. Pharm. Sci.* (1991) 80: 533-538

(22) Shu XZ, Zhu KJ and Song W. Novel pH-sensitive citrate cross-linked chitosan film for drug controlled release. *Int. J. Pharm.* (2001) 212: 19-28

(23) Kemp W. *Infrared Spectroscopy*, 3rd ed. McMillan Press, London (1991) 58-88