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Optimization of chitosan films as a substitute for animal and human epidermal sheets for *in vitro* permeation of polar and non polar drugs

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The present investigation is aimed at preparing chitosan films capable of simulating the flux of modal drugs, 5-fluorouracil (5-FU) and indomethacin (INDO), across rat, rabbit and human cadaver epidermal sheets. Application of statistical design revealed that the concentration of chitosan, crosslinking time and concentration of crosslinking agent significantly influenced the *in vitro* flux of 5-FU and INDO across chitosan films. Multiple linear regression revealed a linear influence of all these active variables on 5-FU and INDO flux. It was deduced from atomic absorption spectroscopic analyses, DSC and IR spectroscopic data that 5% (*m/V*) sodium tripolyphosphate (NaTPP) produced optimum crosslinking of chitosan films. The *in vitro* permeation of both 5-FU and INDO across optimized film formulations was found to be comparable to that obtained across rat, rabbit and human epidermal sheets. These results indicate that optimized chitosan films have a potential to be developed as a substitute for animal and human cadaver epidermal sheets for preliminary *in vitro* permeation studies.

Keywords: chitosan film formulations, sodium tripolyphosphate, rat epidermis, rabbit epidermis, human epidermis

Screening of transdermal formulations during the preliminary phase frequently requires animal skin samples. But, animal skin differs significantly from human skin due to the differences in thickness, nature of stratum corneum, density of hair follicles and sweat glands (1). This makes extrapolation of *in vitro* data to *in vivo* performance less reliable. In addition, various factors, like race, sex, age and anatomical site of skin, influence the reproducibility as well as reliability of data obtained from *in vitro* experiments. Furthermore, availability of both human cadaver and animal skin is becoming highly restricted.

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These problems can be to a large extent overcome by using artificial films in place of animal skin. Artificial membranes possess a distinct advantage over biological membranes, such as controlled composition, ease of preparation and reproducibility of results (2). Therefore, such films can serve as an alternative to animal skin during *in vitro* testing of transdermal formulations, thereby reducing or even obliterating the use of animal skin in preliminary studies.

Chitosan is a linear polysaccharide, which is reported to form complexes with sodium carboxymethylcellulose, citrates, pectin, acacia, agar, sodium caprylate, stearic acid, glutaraldehyde, sodium tripolyphosphate, lactic acid, malic acid and alginic acid (3–7). These chitosan complexes are insoluble in alkaline buffer. This property of complexed chitosan has been utilized in preparing beads (8, 9), microspheres (10, 11) and artificial films (12–14).

Earlier investigations using chitosan membranes have proved useful in simulating drug permeation across rat epidermis (6). Hence, it can be hypothesized that suitably cross-linked chitosan films could be used for stimulating drug permeation across epidermal sheets obtained from other species as well.

The present investigation employed statistical optimization designs for identifying critical formulation and process variables capable of influencing permeation of 5-fluorouracil (5-FU) and indomethacin (INDO), model polar and non polar drugs, respectively, across formulated chitosan films. This was done with an aim to optimize the active variables for preparing chitosan films capable of simulating the flux of both drugs across rat, rabbit and human cadaver epidermal sheets.

EXPERIMENTAL

Chitosan, 95% deacetylated (Central Institute of Fisheries Technology, India), 5-fluorouracil (Dabur Research Foundation, India), indomethacin (Crystal Pharmaceuticals, India), sodium tripolyphosphate and glacial acetic acid (Loba Chemie, India) were used as received. All other reagents were of analytical grade.

Preparation of epidermal sheets

Animal (rat/rabbit) skin was obtained after sacrificing the animal by administration of excess chloroform for inhalation. Human cadaver skin (male) was obtained post-mortem within 6 h of death from the local medical college after obtaining the consent of the relatives of the deceased. These protocols were approved by the respective institutional ethical committees. Dorsal skin portion of albino Wistar rats or rabbits was shaved with an electrical hair clipper and excised after sacrificing. Human cadaver skin was excised from the abdominal/chest portion. Epidermal sheet was separated by soaking the excised whole skin in phosphate buffer saline (PBS, pH 7.4) containing trypsin (0.1%, *m/V*) for 120 s at 60 °C.

Freshly prepared epidermal sheets were washed with PBS and conditioned under stirring in a receptor solution for 4 h before commencing *in vitro* permeation experiments.

Formulation design and preparation of cross-linked chitosan films

For screening the effect of the process and formulation variables (15) on permeation parameters of 5-fluorouracil and indomethacin, the Plackett-Burman screening design (PBD) was used. PBD is a useful experimental design employed to screen various formulation and process variables for their influence on particular responses by conducting a minimum number of experiments. Various chitosan films were prepared by using low and high levels (designated –1 and +1, respectively) of each variable (X1–X8). The film formulations (P1–P8) prepared according to PBD are summarized in Table I. The active variables (that significantly influence the response) may show their effect/interaction in any part of the experimental domain ranging between their low and high levels. In order to investigate the influence of these active variables (concentration of chitosan, concentration of cross-linking agent and cross-linking time) on the permeation of both drugs in the entire experimental domain, experiments were performed using a central composite design (CCD). Additional films (Table II) were prepared using the 2^3 factorial design (1F–8F), six star design or axial points (1S–6S) and centre point (1C–4C) in order to complete the CCD covering the entire experimental domain. Permeation of both drugs across the films was compared with that obtained across rat/rabbit/human epidermal sheets. Correlation between active variables obtained from CCD and *in vitro* flux of both drugs was done employing Statistica software-5.0 (Sta Soft Inc., USA).

Table I. Screening design for identifying active formulation and process variables influencing the flux of indomethacin (INDO) and 5-fluorouracil (5-FU)

Batch No.	X1 (%, *m/V*)	X2 (%, *m/V*)	X3 (min)	X4 (h)	X5 (%, *V/V*)	X6	X7	INDO flux ($\mu g\ h^{-1}\ cm^{-2}$)[a]	5-FU flux ($mg\ h^{-1}\ cm^{-2}$)[a]
P1	+1 (4)	+1 (10)	+1 (45)	–1 (24)	–1 (3)	+1	–1	50.4 ± 12.4	1.045 ± 0.046
P2	–1 (2.5)	+1 (10)	+1 (45)	+1 (48)	+1 (20)	–1	–1	142.7 ± 5.6	2.947 ± 0.045
P3	–1 (2.5)	–1 (5)	+1 (45)	+1 (48)	–1 (3)	+1	+1	44.8 ± 3.6	2.022 ± 0.010
P4	+1 (4)	–1 (5)	–1 (15)	+1 (48)	+1 (20)	+1	–1	64.5 ± 0.2	1.853 ± 0.003
P5	–1 (2.5)	+1 (10)	–1 (15)	–1 (24)	+1 (20)	+1	+1	214.0 ± 0.2	3.280 ± 0.008
P6	+1 (4)	–1 (5)	+1 (45)	–1 (24)	+1 (20)	–1	+1	56.4 ± 2.7	0.699 ± 0.014
P7	+1 (4)	+1 (10)	–1 (15)	+1 (48)	–1 (3)	–1	+1	88.0 ± 0.5	1.851 ± 0.002
P8	–1 (2.5)	–1 (5)	–1 (15)	–1 (24)	–1 (3)	–1	–1	141.5 ± 5.1	2.416 ± 0.097

X1 – concentration of chitosan, X2 – concentration of NaTPP, X3 – crosslinking time, X4 – drying time at 45 °C, X5 – concentration of acetic acid, 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.

All the chitosan films were prepared by the following method. Solution of chitosan (2.5–4%, *m/V*) was prepared in acetic acid (3–20%, *m/V*) and homogenized (2000 rpm). It was filtered through muslin cloth to remove debris and a portion (15 mL) was degassed under vaccum. This solution was cast into a glass ring (cross sectional area 3.14 cm^2) fitted on a polycarbonate Petri dish and subjected to drying. The dried membranes (45 °C

for 24–48 h) were stored in polyethylene bags until used. Cross-linking of chitosan films was done by dipping in a sodium tripolyphosphate (NaTPP) solution (10 mL of 1–20%, *m/V*, for 15–45 min). These films were washed with water to remove excess NaTPP. Freshly crosslinked membranes that were insoluble in the receptor solution (phosphate buffer pH 7.4) for more than 48 h were used for *in vitro* permeation experiments.

Table II. Central composite design using active formulation and process variables influencing the flux of 5-fluorouracil (5-FU) and indomethacin (INDO)

Batch No.	X1 (%, *m/V*)	X2 (%, *m/V*)	X3 (min)	5-FU flux (mg h^{-1} cm^{-2})[a]	INDO flux (μg h^{-1} cm^{-2})[a]
1F	–1 (2.5)	–1 (5)	–1 (15)	2.416 ± 0.097	141.5 ± 5.1
2F	+1 (4)	–1 (5)	–1 (15)	1.853 ± 0.002	64.5 ± 0.2
3F	–1 (2.5)	+1 (10)	–1 (15)	3.280 ± 0.008	214.0 ± 0.2
4F	+1 (4)	+1 (10)	–1 (15)	1.851 ± 0.002	88.0 ± 0.5
5F	–1 (2.5)	–1 (5)	+1 (45)	2.023 ± 0.010	44.8 ± 3.6
6F	+1 (4)	–1 (5)	+1 (45)	0.699 ± 0.014	56.4 ± 2.7
7F	–1 (2.5)	+1 (10)	+1 (45)	2.947 ± 0.045	142.7 ± 5.5
8F	+1 (4)	+1 (10)	+1 (45)	1.045 ± 0.046	50.4 ± 1.2
1S	–1.682 (2)	0 (7.5)	0 (30)	2.894 ± 0.050	168.3 ± 2.3
2S	+1.682 (4.5)	0 (7.5)	0 (30)	1.341 ± 0.056	55.7 ± 0.7
3S	0 (3.25)	–1.682 (3.3)	0 (30)	1.589 ± 0.023	63.5 ± 0.6
4S	0 (3.25)	+1.682 (11.7)	0 (30)	2.921 ± 0.090	160.6 ± 4.9
5S	0 (3.25)	0 (7.5)	–1.682 (5)	3.942 ± 0.069	134.7 ± 7.3
6S	0 (3.25)	0 (7.5)	+1.682 (55.2)	1.658 ± 0.044	52.5 ± 1.1
1C	0 (3.25)	0 (7.5)	0 (30)	2.320 ± 0.006	98.4 ± 1.4
2C	0 (3.25)	0 (7.5)	0 (30)	2.310 ± 0.012	98.5 ± 1.3
3C	0 (3.25)	0 (7.5)	0 (30)	2.314 ± 0.009	98.6 ± 1.2
4C	0 (3.25)	0 (7.5)	0 (30)	2.310 ± 0.012	98.3 ± 1.4

For X1 – X3 see Table I. F – factorial design, S – star design, C – centre points
[a] Values represent mean ± SD of 5 experiments.

Physicochemical characterization of films

Atomic absorption spectroscopy for Na^+ in chitosan films. – Chitosan films were prepared by dissolving chitosan (4%, *m/V*) in 15 mL of 3% (*V/V*) acetic acid. They were dried at 45 °C for 24 h and cross-linked by dipping in 10 mL NaTPP solution (1, 5, 10 or 20%, *m/V)* for 45 min. Each cross-linked film was dissolved 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 G3 filter. The filtrate obtained was subjected to flame atomic absorption spectroscopy for estimation of Na^+ (GBC, 932AAS, Australia).

Infrared absorption spectroscopy. – The cross-linked chitosan films as prepared for atomic absorption spectroscopy were dried to constant mass at 45 °C. Then they were triturated with an equal quantity of KBr and compressed to obtain discs for IR analysis. The spectra of these discs were recorded on a Perkin Elmer RXI, IR spectrophotometer (USA) in the spectral region of 500 to 4000 cm^{-1}.

Differential scanning calorimetry. – Cross linked films (as prepared for IR spectroscopy) were also subjected to DSC studies. Samples of chitosan powder and cross-linked films were stored in a desiccator at 50% RH for 48 h. Then they were subjected to DSC analysis (Mettler Toledo Star System, 821E, Switzerland) employing a heating rate of 10 °C min^{-1}.

In vitro *permeation studies*. – A vertical Franz diffusion cell apparatus was designed and fabricated in our laboratory. It consisted of 8 glass diffusion cells (20 mL each). Stirring of the receptor fluid in each cell was done by magnetic stirrers (300 rpm) at a temperature of 37 ± 0.5 °C by a water heating system. Chitosan membranes or epidermal sheets were clamped between donor and receptor compartments. The receptor compartment contained phosphate buffer (pH 7.4), sodium azide (0.5%, *m/V*) and PEG 400 (5.0%, *V/V)*. Each 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 × 250 mm) and UV detector (2487 Dual wavelength). Sodium acetate (0.1%, *m/V*) and methanol/citrate buffer 10 mmol L^{-1} (75:25), at flow rates of 0.6 mL min^{-1} and 1.0 mL min^{-1}, were used as mobile phases for 5-FU and INDO, respectively. The respective detection wavelengths were 265 and 240 nm, as reported by Sasaki *et al*. (16).

Statistical analysis

All possible pairs consisting of the means of T_m (peak transition temperature), ΔH (enthalpy of transition) or *in vitro* flux were subjected to the *t*-test to test the difference at 5% level of significance.

RESULTS AND DISCUSSION

Preliminary studies were carried out to evaluate the influence of the formulation and process variables on the permeation of 5-FU and INDO across NaTPP cross-linked chitosan films. Data on permeation through chitosan films formulated according to the Plackett-Burman design revealed that the concentration of chitosan, cross-linking time and concentration of cross linking agent influenced the flux (slope of the linear portion of the cumulative amount permeated *vs.* time) of both 5-FU (model polar drug) and INDO (model non polar drug). These data are summarized in Table I. The effect of various formulation and process variables (X1…X7) on 5-FU flux (Y1) across chitosan films was found to be represented by the equation: Y1 = 2.0143 – 0.6521X1 + 0.2665X2 – 0.3359X3 + 0.1542X4 + 0.1806X5 + 0.036X6 – 0.051X7 and that for INDO flux (Y2) by the equation: Y2 = 100.2 – 35.46X1 + 23.48X2 – 26.71X3 – 15.29X4 + 19.113X5 – 6.86X6 + 0.513X7. Three fac-

tors that were found active from the PBD were further studied by preparing additional formulations (Table II) using the central composite design. Multiple linear regression of the data obtained from CCD revealed that the influence of all the active variables on the flux of both drugs was independent of each other (15). The data show that the flux of 5-FU and INDO decreased linearly with an increase in chitosan concentration and cross-linking time (low level to high level). However, the flux was found to increase with an increase in the concentration of the cross-linking agent (Tables I and II).

The unexpected increase of 5-FU and INDO flux with increasing the concentration of the cross-linking agent seems to be due to the influence of the negative charge developed on chitosan films as a consequence of cross-linking with sodium tripolyphosphate. Chitosan in solid state contains amino groups. When dissolved in acetic acid, the $-NH_2$ groups get converted to $CH_3COO^- NH_3^+-$. Therefore, chitosan itself, when dissolved in acetic acid, develops a net positive charge (5). It is logical to expect that upon cross-linking, CH_3COO^- ions present in chitosan films will be replaced with NaTPP (cross-linking agent).

Atomic absorption spectroscopy data show that an increase in NaTPP concentration during cross linking increased the Na^+ content (the counter ion) in cross-linked chitosan films (Table III). It seems that at a low NaTPP concentration, the crosslinking was of the $-NH_3^+ {}^-OPO_2 - PO_2^- - O_2PO^- {}^+ NH_3-$ type. Thus, with an increase in NaTPP concentration, the net charge on cross-linked films is expected to shift from positive to negative due to the attachment of $-PO_3^-$ moieties. Availability of extra $CH_3COO^- NH_3^+-$ groups in the chitosan molecule seems to result in linkage of two terminal $-PO_3^-$ moieties of the NaTPP molecule with two $CH_3COO^- NH_3^+-$ moieties of two chitosan monomers, one on each side. As the concentration of NaTPP increased, only one terminal $-PO_3^-$ moiety got attached to one $CH_3COO^- NH_3^+-$ moiety of each chitosan monomer. This, perhaps, altered the previous linkage to $-NH_3^+ {}^-OPO_2 - PO_2^- - O_2PO^- {}^+Na$. This explanation was supported by the findings of IR spectroscopy. The flux of 5-FU and INDO across films crosslinked with 1% (*m/V*), NaTPP was found to be the highest. This could be attributed to improper crosslinking at a low NaTPP concentration. Increase in NaTPP concentration (5%, *m/V*) is expected to decrease the positive charge on chitosan film. However, it can be envisaged to be still high enough to interact with and restrict the movement of

Table III. Physicochemical characteristics of chitosan films (4%, m/V*) cross-linked with different concentrations of NaTPP*

NaTPP (%, *m/V*)	Na^+ (μg per film)[a]	5-FU flux (mg h^{-1} cm^{-2})[a]	INDO flux (μg h^{-1} cm^{-2})[a]	DSC analysis[a]			
				First endotherm		Second endotherm	
				T_m (°C)	ΔH (J g^{-1})	T_m (°C)	ΔH (J g^{-1})
1	6.3 ± 0.1	2.912 ± 0.006	98.4 ± 0.6	79.8 ± 1.2	80.8 ± 1.2	228.6 ± 8.7	39.5 ± 3.1
5	44.6 ± 5.6	0.683 ± 0.003	50.4 ± 4.4	55.3 ± 1.2	1.9 ± 2.3	220.3 ± 2.3	168.9 ± 2.0
10	46.0 ± 1.3	1.107 ± 0.010	63.4 ± 3.2	63.6 ± 1.1	94.3 ± 3.2	244.8 ± 8.9	55.1 ± 5.6
20	60.3 ± 2.2	2.651 ± 0.008	88.0 ± 0.5	59.7 ± 1.5	86.0 ± 3.3	243.8 ± 9.3	52.1 ± 9.3

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

negatively charged 5-FU and INDO species. Therefore, this interaction seems to be responsible for decreasing 5-FU and INDO flux across chitosan films crosslinked with 5% (*m/V)* NaTPP to a minimum value. Hence, crosslinking with 5% (*m/V)*, NaTPP appears to indicate the most useful crosslinking of chitosan with NaTPP. However, further increase in NaTPP concentration (10 and 20%, *m/V*) resulted in an enhanced passage of 5-FU and INDO across the films. This might be due to the increased negative charge on the film (due to excessive presence of $-PO_3^-$ moieties) that repelled the negatively charged 5-FU-/INDO molecules across the film. It is important to note that Remunan-Lopez and Bodmeier (14) observed the diffusion of chlorpheniramine maleate (a basic drug) that is positively charged in chitosan films to decrease with an increase in NaTPP concentration. Therefore, enhanced permeation of both 5-FU and INDO across chitosan films cross--linked with an increasing concentration of NaTPP can be ascribed to their acidic nature due to which both drugs will be negatively charged in the films and hence, repelled into the receptor compartment. Therefore, these findings are in agreement with those of Remunan-Lopez and Bodmeier (14). Hence, the overwhelming role of the concentration of the crosslinking agent in controlling the permeation of 5-FU and INDO across chitosan films was evident.

The IR spectra showed peaks at 1060–1300 cm^{-1}, suggesting the presence of phosphonate linkage between $-NH_3^+$ of chitosan and $-PO_3^-$ moieties of NaTPP during the crosslinking process. Only one peak at 1120 cm^{-1} was observed in the film when crosslinked with 1% (*m/V)* NaTPP, indicating no appreciable linkage. However, the films cross-

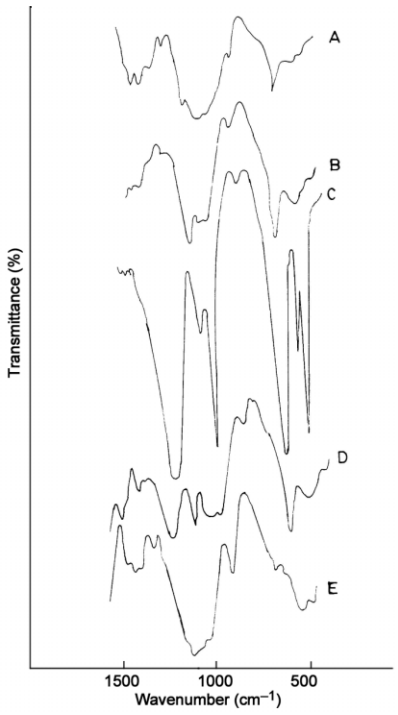

Fig. 1. IR spectra of: A) chitosan powder, and chitosan (4%, *m/V*) films cross-linked with NaTPP (%, *m/V*): B) 1, C) 5, D) 10 and E) 20.

linked by 5%, *m/V*, NaTPP showed two peaks, at 1140 cm^{-1} and at 1280 cm^{-1} (Fig. 1), indicative of symmetric and antisymmetric stretching of phosphonate linkage, respectively. The latter peak (antisymmetric) is known to occur due to restricted rotation (17). Therefore, the two terminal $–PO_3^-$ moieties of the NaTPP molecule seem to be linked with two $CH_3COO^–$ NH_3^+– moieties of two chitosan monomers, one on each side. This crosslinking perhaps restricted the permeation of drug molecules and resulted in the lowest flux of both drugs across films crosslinked with 5% (*m/V)* NaTPP. These two peaks were also observed in films crosslinked with 10% *(m/V)* NaTPP. However, the intensity of the antisymmetric peak was significantly reduced. This antisymmetric peak was found to merge into a single broad band at 1060 cm^{-1}, indicating the absence of restricted rotation in films crosslinked with 20% (*m/V)* NaTPP. In consonance, greater permeation of both drugs was observed across films crosslinked with either 10 or 20% (*m/V)* NaTPP.

DSC thermograms of all the crosslinked films exhibited two endotherms (Fig. 2). The first endotherm obtained below 100 °C could be attributed to water loss since all the films were saturated in 50% RH prior to thermal analysis. A statistically significant difference at 5% level of confidence was observed for the second endothermic T_m values of films cross-linked with 1, 5 or 10 (*m/V*) NaTPP. T_m values followed the order: 10 ~ 20 > 1 > 5% (*m/V*) NaTPP. ΔH for this endotherm was found to be 39.47, 168.93, 55.06 and 52.09 J g^{-1} for films cross-linked with 1, 5, 10, 20% *m/V* NaTPP, respectively. Significantly different were the ΔH values for films cross-linked with 1 or 5 (*m/V*) NaTPP. No significant difference was detected for either T_m or ΔH values of films cross-linked with 10 and 20%

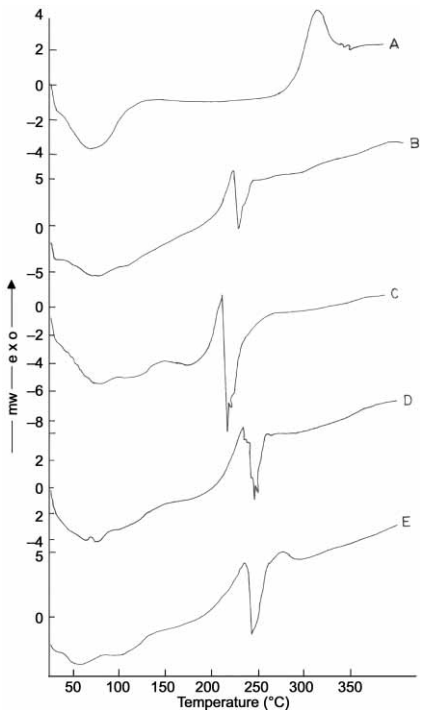

Fig. 2. DSC thermograms of: A) chitosan powder, and chitosan (4%, *m/V*) films cross-linked with NaTPP (%, *m/V*): B) 1, C) 5, D) 10 and E) 20.

(*m/V*) NaTPP. It is noteworthly that different concentrations of NaTPP significantly influenced the *in vitro* flux of both 5-FU and INDO across films (Table III). *In vitro* flux values followed the order: 1 > 20 > 10 > 5% (*m/V*) NaTPP. Hence, the cross-linking of chitosan by 5% (*m/V*) NaTPP (reflected in the highest ΔH value of the second endotherm) appears to result in the lowest permeation of both drugs.

The three equations generated using the statistical software for both drugs (Table IV) were solved to calculate the optimum values of X1 (concentration of chitosan), X2 (concentration of crosslinking agent) and X3 (crosslinking time) for preparing chitosan films that will exhibit a flux comparable to that across epidermal sheets. The lower and higher solved values of all the active variables (X1, X2, X3) summarized in Table IV 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 V shows that the permeation of both 5-FU and INDO across cross-linked chitosan films formulated using various optimized values of active variables did not differ significantly as compared to that across rat/rabbit/human epidermal sheets.

Table IV. Equations for relating the influence of the concentration of chitosan (X1), cross-linking agent (X2) and cross-linking time (X3) on the flux of 5-FU (Y1) and INDO (Y2) across cross-linked chitosan films

Treatment	Equation	
X1 *vs.* X2	Y1 = 2.206 + 0.573X1 + 0.320X2	Y2 = 101.744 + 34.638X1 + 25.715X2
X2 *vs.* X3	Y1 = 2.206 + 0.320X2 + 0.478X3	Y2 = 101.744 + 25.715X2 + 25.769X3
X3 *vs.* X1	Y1 = 2.206 + 0.573X1 + 0.478X3	Y2 = 101.744 + 34.638X1 + 25.769X3

	Optimized variable for films simulating the flux of					
	5-FU			INDO		
Epidermis type	X1 (%, *m/V*)	X2 (%, *m/V*)	X3 (min)	X1 (%, *m/V*)	X2 (%, *m/V*)	X3 (min)
Rat	3.00–3.25	6.3–7.5	25–30	3.12–3.18	7.0–7.2	26.6–28.0
Rabbit	3.44	8.7	35	4.40–4.60	12.7–13.5	62.0–66.0
Human	2.20–2.30	1.5–1.8	6–8	2.50–2.55	4.1–4.3	9.5–11.3

It is important to note that the optimized values for preparing chitosan films capable of simulating the flux of either drug are different for epidermal sheets of different animals. This indicates that a single chitosan film with a particular optimized composition cannot be used for simulating drug permeation across all types of epidermis. This is because of the different inherent permeabilities of the epidermis of different animals due to variation in their biochemical constituents, anatomical ultrastructure, *etc*. Nevertheless, a high correlation evident from Fig. 3 and Fig. 4 suggests that NaTPP cross-linked chitosan films prepared by using their optimized composition can be used to simulate the *in vitro* permeation of 5-FU and INDO across rat, rabbit and human cadaver epidermal sheets.

Table V. Optimized composition of cross-linked chitosan films capable of mimicking the 5-FU and INDO flux across rat, rabbit and human epidermis

Animal epidermis		Optimized films	Statistical difference
Type	Flux[a] (epidermal sheet)	Flux[a]	
5-Fluorouracil (mg h^{-1} cm^{-2})			
RAT	2.05 ± 0.15	2.13 ± 0.66	NS
Rabbit	2.47 ± 0.03	2.70 ± 0.94	NS
Human	0.72 ± 0.03	0.76 ± 0.23	NS
Indomethacin (μg h^{-1} cm^{-2})			
Rat	92.6 ± 2.5	97.23 ± 0.56	NS
Rabbit	217.6 ± 7.5	220.60 ± 0.52	NS
Human	34.2 ± 3.2	31.66 ± 0.13	NS

Comparison between epidermal sheets and optimized films: NS – no statistically significant difference.
[a] Values represent mean ± SD of 5 experiments.

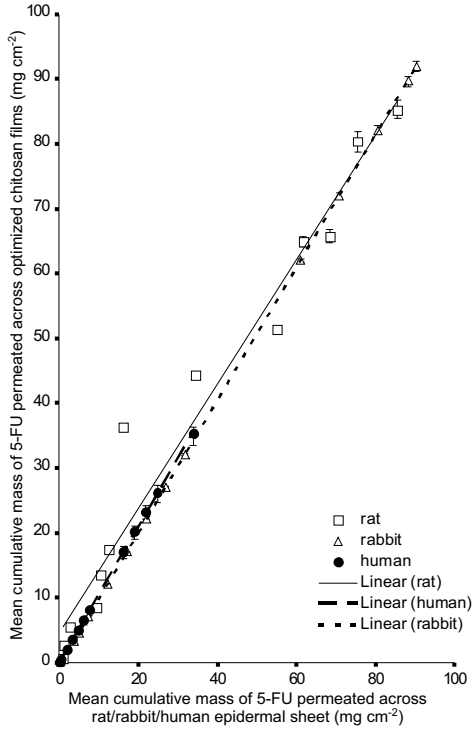

Fig. 3. Correlation between *in vitro* permeation of 5-FU across optimized chitosan films and rat epidermal sheet (Y = 0.956 X + 4.682, R^2 = 0.96), rabbit epidermal sheet (Y = 1.0225 X – 0.4292, R^2 = 1.00) and human cadaver epidermal sheet (Y = 1.048 X + 0.097, R^2 = 0.99). Each print values mean ± SD, n = 5.

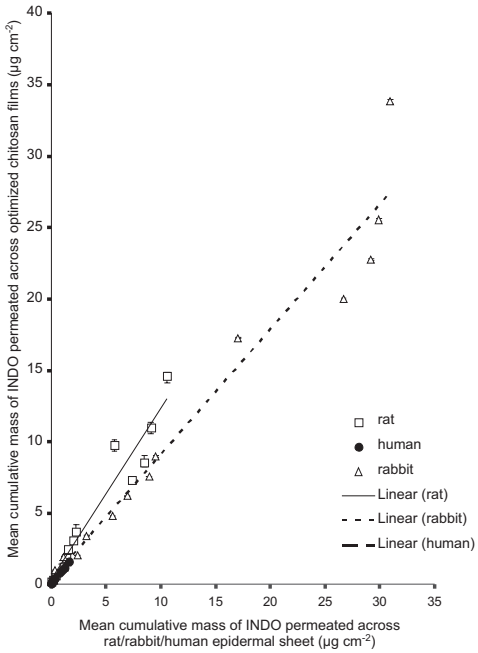

Fig. 4. Correlation between *in vitro* permeation of INDO across optimized chitosan films and rat epidermal sheet (Y = 1.194 X + 0.382, R^2 = 0.94), rabbit epidermal sheet (Y = 0.8758 X + 0.372, R^2 = 0.95), and human cadaver epidermal sheet (Y = 0.932 X + 0.1336, R^2 = 0.99). Each print values mean ± SD, n = 5.

CONCLUSIONS

NaTPP may critically influence the permeation of both hydrophilic and lipophilic drugs across chitosan films. The findings of *in vitro* permeation studies were explained on the basis of the contribution of charge to the film by increasing concentration of NaTPP and the evidence obtained from atomic absorption spectroscopy, IR spectroscopy and DSC analysis of cross-linked films. Further, the results suggest that the formulation and process variables can be modified to prepare NaTPP cross-linked chitosan films that simulate the *in vitro* permeation of polar and non polar drug species across animal and human epidermal sheets. This knowledge has a great potential for exploitation in transdermal dosage form research for making films as an alternative to animal and human skin. This is expected to reduce the widespread use of natural skin during preliminary *in vitro* investigations on transdermals.

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S A Ž E T A K

Optimizacija kitozanskih filmova kao zamjena za životinjsku i humanu epidermu za *in vitro* permeaciju polarnih i nepolarnih lijekova

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U radu je opisana priprava kitosanskih filmova pogodnih za simulaciju prijelaza modelnih lijekova, 5-fluorouracila (5-FU) i indometacina (INDO), kroz epidermalne slojeve štakora, zeca i čovjeka. Koncentracija kitosana, vrijeme umrežavanja i koncentracija reagensa za umrežavanje značajno su utjecale na *in vitro* prolaz 5-FU i INDO kroz kitosanske filmove. Multiplom linearnom regresijom pokazano je da sve navedene varijable imaju linearni utjecaj na prolaz 5-FU i INDO. Uz pomoć atomske apsorpcijske spektralne analize, DSC i IR spektroskopskih podataka zaključeno je da je 5%-tna (*m/V*) otopina

natrijevog tripolifosfata (NaTPP) optimalna za umrežavanje kitosanskih filmova. Pronađeno je da je *in vitro* permeacija 5-FU i INDO kroz optimiziranu formulaciju kitozanskog filma usporediva s permeacijom kroz epidermalne slojeve štakora, zeca i čovjeka. Rezultati upućuju na to da se optimizirani kitozanski filmovi mogu upotrijebiti kao nadomjestak animalne i humane epiderme u preliminarnim *in vitro* permeacijskim istraživanjima.

Ključne riječi: kitosanski filmovi, natrijev tripolifosfat, epiderma štakora, epiderma zeca, humana epiderma

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