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"FORMULATION AND EVALUATION BASED ETDDS AGAINST ALZHEIMER'S DISEASE USING THE NATURAL POLYMER (PAAm-g-Dxt)

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ABSTRACT

In this work, we have synthesized novel electrically-responsive graft copolymers were synthesized by the method of polymerization by free radical initiation under the nitrogen environment, further; they were made electrically responsive by alkaline hydrolysis process. Further, the electro-sensitive transdermal drug delivery systems (ETDDS) loaded with Galantamine were developed forthe treatment of Alzemier's disease using graft copolymer as drug reservoir, crosslinked graft copolymer-polyvinyl alcohol composite films as RCM andpoly (styrene) film as backing membranes.

In the second part of the study, we have synthesized hydrolyzed PAAm-g-Dxt by polymerization under the nitrogen atmosphere. The copolymer characterizations were 1H-NMR, FTIR, NE, elemental analysis and TGA to endorse the grafting. Further, we developed an ETDDS utilizing the PAAm-g-Dxt for drug delivery through skin. Without electric stimulus, a little amount of drug got permeated from the ETDDS; the drug permeation got increased considerably under applied electric stimulus.

Key words: Galantamine, Graft Copolymers, Morris water maze test, Alzemier's disease

INTRODUCTION

Alzheimer's disease is characterized by the impairment of cholinergic function. One hypothesis is that this impairment contributes to the cognitive deficits caused by the disease. This hypothesis forms the basis for use of galantamine as a cholinergic enhancer in the treatment of Alzheimer's. Galantamine inhibits acetylcholinesterase, an enzyme which hydrolyzes acetylcholine. As a result of acetylcholinesterase inhibition, galantamine increases the availability of acetylcholine for synaptic transmission. Additionally, galantamine binds to the allosteric sites of nicotinic receptors, which causes a conformational change. This allosteric modulation increases the nicotinic receptor's response to acetylcholine. The activation of presynaptic nicotinic receptors increases the release of acetylcholine, further increasing the availability of acetylcholine. Galantamine's competitive inhibition of acetylcholinesterase and allosteric nicotinic modulation serves as a dual mechanism of action.[1]

To reduce the prevalence of negative side effects associated with galantamine, such as nausea and vomiting, a dose-escalation scheme may be used. The use of a dose-escalation scheme has been well accepted in countries where galantamine is used. A dose-escalation scheme for Alzheimer's treatment involves a recommended starting dosage of 4 mg galantamine tablets given twice a day (8 mg/day). After a minimum of 4 weeks, the dosage may then be increased to 8 mg given twice a day (16 mg/day). After a minimum of 4 weeks at 16 mg/day, the treatment may be increased to 12 mg given twice a day (24 mg/day). Dosage increases are based upon the assessment of clinical benefit as well as tolerability of the previous dosage. If treatment is interrupted for more than three days, the process is usually restarted, beginning at the starting dosage, and re-escalating to the current dose. It has been found that a dosage between 16–24 mg/day is the optimal dosage[2].

Galantamine also works as a weak competitive and reversible cholinesterase inhibitor in all areas of the body. By inhibiting acetylcholinesterase, it increases the concentration and thereby action of acetylcholine in certain parts of the brain. Galantamine's effects on nAChRs and complementary acetylcholinesterase inhibition make up a dual mechanism of action. It is hypothesized that this action might relieve some of the symptoms of Alzheimer's[3].

MATERIAL AND METHOD

Galantamine were obtained from Sun Pharma Ltd., Mumbai, Dextran, Pectin, Pullulan, Sodium hydroxide (NaOH) Pallets, Potassium dihydrogen ortho phosphate (KH_2PO_4), Sodium dihydrogen ortho phosphate (NaH_2PO_4) were obtained from HiMedia Lab. Mumbai. Inulin obtained from Central Drug House, New Delhi. Ammonium Persulphate (APS) were obtained from Fisher Scientific, Mumbai. Glutaraldehyde obtained from S.D.fine Chemicals Ltd., Mumbai. Hydrochloric acid (HCL) were obtained dorm Central Drug House.

Synthesis of electrically-responsive graft copolymers

Synthesis of polyacrylamide (PAAm-*g*-Dxt) was done by polymerization through free-radical formation. Two grams of natural polymers namely, dextran, pectin, inulin and pullulan were allowed to soak in hundred ml purified water with nitrogen supply. The round bottom flask was heated at 80 ^{o}C and accurately weighed 0.43 gm of ammonium persulphate (APS) was blended with the reactant solution and stirring was continued for 20 min. Further, to this, 10 ml 0.105 mol acrylamide solution was mixed and reaction was allowed to proceed for 60 min under nitrogen purging. Once the reaction was competing, product was allowed settle at room temperature and then it was blended with methanol (400 ml), further, product was repetitively washed with the help of methanol. Then the graft product was dried at 50 °C whole night and stored in well closed container[4]. The following grafting parameters were determined for the copolymers.

$$\text{Percent AAm grafting} = \frac{\text{Amount of AAm in graft polymer}}{\text{Amount of the graft polymer}} \text{ X } 100$$

$$Precent\ \ grafting\ efficiency = \frac{amount\ of\ graft\ copolymer - amount\ of\ substrate}{amount\ of\ monomer} \times 100$$

Hydrolysis of grafted copolymers

After dissolving two gm of PAAm-*g*-Dxt copolymers in hundred ml NaOH solution (0.9 M strength), the solution was heated at 75 °C with constant stirring for sixty min and then the it was to room temperature; excess methanol was added, filtered and dried at 50 °Covernight. Finally, the dried product was taken out and stored properly[5].

Characterization of grafted copolymers

The synthesized copolymers were characterized using following techniques;

i) ^{1}H-NMR analysis, ii) FTIR spectroscopy, iii) Elemental analysis, iv) Neutralizationequivalent values, v) Thermogravimetric analysis and vi) Determination of viscosity

1H-NMR spectroscopy

^{1}H-NMR analysis was conducted on monomer (AAm), native polymers (PCT), and grafted copolymers (PAAm-*g*-Dxt). The 10 mg/dm^{3} of samples were dissolved in $D_2O$and spectra were recorded on NMR spectrometer (Bruker Avance III, 400MHz, Germany).

FTIR spectroscopy

Native polymers (PCT), polyacrylamide-grafted- copolymers (PAAm-*g*-Dxt) and alkaline hydrolyzed polyacrylamide-grafted-copolymers (PAAm-*g*-Dxt) were mixed with potassium bromide at 600 kg to get pellets. Then the spectra between 400 and 4000 cm^{-1}were recorded using 8400S Shimadzu FTIR spectrophotometer[6'7].

Elemental analysis

The native polymers (Dxt), polyacrylamide-grafted- copolymers (PAAm-*g*-Dxt)and alkaline hydrolyzed polyacrylamide-grafted-copolymers (PAAm-*g*-Dxt) were analyzed for estimation of percentages of N, C, H with the help of Elementar Vario EL III CHN analyzer.

Determination of neutralization equivalent values

Neutralization equivalent values were assessed with titration method using standard base. The two hundred milligrams of native polymers (Dxt), polyacrylamide-grafted-copolymers (PAAm-*g*-Dxt) and alkaline hydrolyzed polyacrylamide-grafted- copolymers (PAAm-*g*-Dxt) were mixed with to 0.1 N HCl and kept for 6 h, the excess hydrogen ions were determined by back titration utilizing standard 0.1 N NaOH. Then the equivalent mass of carboxylic acid groups was calculated[8].

Thermogravimetric analysis

Thermogravimetric analysis of native polymers (Dxt), polyacrylamide-grafted-copolymers (PAAm-*g*-Dxt) was done at 10 °C/min under dynamic argon gas flowing at 50 ml/min, in the temperature range of 40-400°C. The analysis was done with the help of Diamond TG/DTA, Perkin Elmer thermal analyzer.

Viscosity measurement

The viscosity of solutions of native polymers (PCT), polyacrylamide-grafted-copolymers (PAAm-*g*-Dxt) and hydrolyzed polyacrylamide-grafted-copolymers (PAAm-*g*-Dxt) was assessed using Brookfield, RVDV-E Viscometer.

Development of electrically-responsive transdermal drug delivery systems

The fabrication of electrically-responsive transdermal drug delivery systems (ETDDS) involved two steps; i) preparation of hydrogel reservoirs and ii) preparation of rate controlling membranes (RCMs)[121][8].

Hydrogel reservoirs

For the preparation of hydrogel reservoir, accurately weighed quantity of different graft co-polymers (PAAm-*g*-Dxt) were dissolved in purified water and stirred on magnetic stirrer till homogenous solution was formed. Then the accurately weighed quantities of methylparaben and drugs were mixed with the above polymeric solution while stirring. At the end, various concentrations of glutaraldehyde (GA) along with 0.1 N HCl (0.5 ml) was mixed with polymer solution and stirring was continued for 30 min. The prepared reservoirs were stored properly preparation the ETDDS[122][9].

RCMs

The RCMs were prepared using different polymers namely; pectin, dextran, inulin and pullulan. These polymers were added to purified water and stirred well on magnetic stirrer till homogeneous solution was formed. The different proportions of polyvinyl alcohol (PVA) and Polyethylene 200 (PEG 200) were blended with the above polymer solution with stirring. Finally, this polymeric solution was transferred to glass ring fixed on a glass plate. After 24 hours, the dried films were separated from the glass plate. Further, these membranes were cross-linked in methanol solutionwith required quantities of GA and 0.1 N HCl for 2 hours at 50 ^{o}C and then dried for 24 hrs. Further, the ETDDS were prepared by sandwiching the accurately weighed quantity of hydrogel reservoir between RCM and backing membranes (2 cm^2) and heat sealed to get leak proof devices[10]. The

formulation compositions are given in Tables 1.

Table 5.Formulation details of PAAm-g-Dxt based ETDDS

Code	Hydrogel reservoir					RCMs				
	PAAm-g-Dxt (%W/V)	Drug (%w/w)*	GA (%w/w)*	0.1N HCL (ml)	Methyl Paraben (mg)	Dextran (% w/v)	Polyvinyl alcohol (% w/v)	Polyethylene glycol 200 (% w/w)	GA (% w/w)*	0.1 N HCl (ml)
Dxt 1	3	20	3	0.5	15	2	2	10	3	0.5
Dxt 2	4	20	3	0.5	15	2	2	10	3	0.5
Dxt 3	5	20	3	0.5	15	2	2	10	3	0.5
Dxt 4	5	20	6	0.5	15	2	2	10	3	0.5
Dxt 5	5	20	9	0.5	15	2	2	10	3	0.5
Dxt 6	5	20	9	0.5	15	2	2	10	6	0.5
Dxt 7	5	20	9	0.5	15	2	2	10	9	0.5
Dxt 8	5	20	9	0.5	15	2	2	10	12	0.5

Evaluation of hydrogel reservoirs

Drug content

The required quantity of PAAm-*g*-Dxt reservoir gel (1gm) were transferred to 80 ml of buffer of pH 7.4 and set aside overnight. Then this solution was moderately heated at 40 ^{o}C for about 10 min, filtered and volume was made up to 100 ml using pH 7.4 buffer. The absorbance was recorded at 290 nm using UV-VIS spectrophotom[11].

Determination of pH

One gm of gel reservoir was mixed with 10 ml purified water and the pH of the solution was measured using pen pH meter.

Evaluation of RCMs

Thickness uniformity

Digital micrometer (Mitutoyo, Japan) was used to measure the thickness of RCMs. The mean of the three observations was considered for calculation.

Water vapor transmission (WVT) studies

The transmission cells were rinsed with water and dried. The two gm of calcium chloride (fused) was added to cell and RCMs were fixed over brim. Theinitial weight of the cell was noted and then placed in the firmly sealed desiccator having saturated KCl solution. The humidity recorded

was 82% RH. The cell was taken out and weights were recorded at different times up to24 h. The rate of WVTwas calculated from increase in the weights using the equation,

WVT=WS / L ----------(3)

W = water vapor transmitted in gm, L = thickness, S = exposed surface area It is represented as the number of grams of water transmitted/hr.sqcm.

The tensile strength of RCMs was recorded with Hounsfield, Slin fold Universal testing machine, which was fitted with two loading jaws; the upper one was movable and lower one was fixed. The prepared RCMs were cut into the rectangular patches of size 2 x 0.8 cm^2 and fixed between two jaws. Upper jaw was allowed to move at 100 mm/min and force was applied (using 10 kg load cell) gradually till the RCM was broken. The tensile strengths (Kg) and extension value (mm) of RCMs were read directly from the dial reading[11].

Scanning electron microscopic study

The RCMs were coated with platinum and examined at acceleration voltage of 10-20 kV and suitable magnifications under scanning electron microscope (JEOL, JSM-6360, Kyoto and Japan).

Differential scanning calorimetry

The DSC thermograms of RCMs were recorded for 5-10 mg samples at 10 K/min between 20 ^oC and 300 ^oC temperature under nitrogen flow rate of 25 ml/min. The samples were wrapped in aluminum crucibles closed with perforated lids and an empty crucible was kept as reference[12].

X-Ray diffraction studies

The difractograms were measured using the instrument, Philips, PW -171. For internal standard and calibration, the quartz was used. The instrument was connected to digital assembly through computer and data was recorded in the 2θ range 0-80^o.

In-vitro permeation of drug from ETDDS

"The hair of the abdominal area of healthy wistar rats weighing between 150- 200 g was cautiously separated without causing injury to the skin and then the skin was excised. The dermal part of skin was carefully cleaned to remove remaining tissue or blood vessel. The ETDDS was put in contact with stratum corneum side of the skin and in turn was secured on the donor compartment of the Keshary-Chien diffusion cell using bonding agent. The study was performed on magnetic stirrer at 32 $^oC\pm$ 5^oC and 100 rpm using pH 7.4 buffer solutions as receptor medium for 24 hours. Then the donor compartment was firmly secured on receptor compartment. A carbon anode was placed in the donor compartment and a carbon cathode was placed in the receptor compartment. The electric stimulus was applied using regulated DC power source. At specific times, 5 ml of samples were taken out and the volume withdrawn was replaced with an equal amount of buffer of pH 7.4". The amount of drug

permeation was determined at 290 nm [13].

This experiment was conducted without electric stimulus, with the electric stimulus and with switching "on–off" stimulus mode of permeation for that refer figure 2.

Figure 1 Photograph showing electric stimulus aided drug permeationexperiment

In vivo studies

Among all the ETDDS prepared using different graft copolymers of or PAAm-g-Pectin formulation prepared using PAAm-g-Inulin has shown adequate *in vitro* performancein terms of drug permeation[14].

Experimental protocol

Male Wistar rats (150-250 g) were obtained from PES College of pharmacy Bangalore. Rats were housed in the cages at 25 ^{o}C with a controlled 12 h light-dark cycles. Food and water were supplied adequately. Studies were performed as per the CPCSEA guidelines.

Rats were separated into four groups of six rats in each group and treated for 15 days as follows; the rats of normal control group received no treatment, disease control group rats received scopolamine (i.p. injection @ 1mg/kg), standard group rats received scopolamine injection (i.p. 1 mg/kg) and unformulated Galantamine (orally @ 2 mg/kg) and test group rats received scopolamine (i.p @ 1 mg/kg) and INU8 transdermal formulation equivalent to 2 mg Galantamine transdermally with 2 mA electric stimulus daily for 45 min. The scopolamine was administered by intra- peritonial injection and unformulated Galantamine was administered orally as sodium carboxymethylcellulose suspension. The abdominal hair of test group rats was removed by electrical clippers and the transdermal formulation PCT 8 was applied on the skin using an adhesive[15]. A carbon anode was fitted into the transdermal formulation; while, carbon cathode was placed elsewhere on the skin separated by a distance of 5 cm.

The circuit was completed by linking electrodes to the respective negative and positive poles of the constant DC power unit. A 2-mA electric current was supplied for a period of 45 minutes. The electro-stimulated drug permeation study was conducted under anesthesia using ketamine 90 mg/kg.

Behavioral studies

Morris water maze test (MWM)

This test represents specific test of spatial memory. The apparatus consists of spherical pond having diameter of 150 cm and height of 45 cm, pond was filled with water at depth of 30 cm at it was maintained at 28±1°C. Small quantity of titanium dioxide was mixed with the water in order to make opaque water. The pond was separated as four equal quadrants and marked by threads. A concealed stage was placed 1 cm down in the center of fourth quadrant. The rats were allowed to go for four successive trials well before the experiments on 7^{th} and 14^{th} day. The rat was put in to the pond facing wall of the tank and made to escape to hidden stand and remain for 20 sec; the escape latency (ELT) was noted. The ELT is termed as time taken by the rat from dropping in to water to escape on the hidden stand (Cut off period was set as 120 sec).[1]

Measurement of biochemical parameters of the brain tissue

On 15^{th} day, after behavioral assessment, the rats were sacrificed by decapitation. The brains were isolated, forebrain was separated out and cerebellum was rejected The brain was rinsed with ice-cold isotonic saline. A tissue homogenate was made in 10% 0.1M buffer of pH 7.5 by centrifuging at 10,000 rpm for 15 min; the supernatant liquid was used for the determination of biochemical parameters like lipid peroxidation (LPO), glutathione (GSH), catalase (CAT) and acetylcholinesterase (AChE) activity[16].

Lipid peroxidation

"The 0.5 ml tissue homogenate was allowed to incubate with trichloroacetic acid (15%), 2-thiobarbituric acid (0.375%) and HCl (5 N) at 95 ºC for 15 minutes. The homogenate was cooled to room temperature and centrifuged". The supernatant liquid was collected and absorbance was recorded at 480 nm on UV-Visspectrophotometer.[17]

Glutathione (GSH)

"The homogenate (5%) was made in 20 mM EDTA solution of pH 4.8. Then the hundred microliter homogenate was mixed with one milliltre 0.2 M Tris-EDTA buffer solution of pH 8.3 and 0.9 ml of 20 mM EDTA (pH 4.8). To this solution, 20 µlEllman's reagents (10 mmol/l DTNB in methanol) was added and kept aside for 30 min for incubation". Then these samples were subjected for centrifugation and absorbance was measured at 395 nm on UV spectrophotometer [18].

Catalase activity (CAT)

"To the 0.05 ml tissues homogenate (10% w/v), 1.95 ml phosphate buffer (0.05 M, pH 7.0), one ml hydrogen peroxide (0.019 M) was added and incubated at room temperature". Further absorbance was recorded at 220 nm on UV-Vis spectrophotometer [19].

Acetylcholinesterase (AChE) activity

"The 0.4 ml of tissue homogenate was added to 2.6 ml of phosphate buffer(pH 8); to this, 0.1 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) reagent was added and mixed properly"; Absorbance was measured at 380 nm using UV-Vis spectrophotometer[19].

Histopathology of brain tissue

For histopathological examination, the brain specimens were stained by fixing in 10% formalin for 24hrs finally washed with water. Dilutions of alcohol were adopted for dehydration. Further, samples were cleaned in xylene, fixed in paraffin at 56 ºC for 24 h and then they were sectioned at 4 microns thickness by microtome. Thestaining was done with hematoxylin & eosin for light microscopy

Immunohistochemistry of brain derived neurotrophic factor (BDNF)

Samples were stained using commercial monoclonal antibodies against the BDNF. The examination slides were heated for the antigen recovery in microwave oven at 600 W and cooled. The antibody dilution was done at 1:140 in phosphate buffer. Antigen-antibody reaction was developed using avidin-biotin complex technique. The samples were incubated with DAB-peroxidase solution and counterstained with Mayer's hematoxylin. Immunohistochemistry examination was performed and images were captured at 40X on Microlumina ultra resolution scanning digital camera[20].

Statistics

One way ANOVA- multiple comparisons Dunnett's test was used for statisticalcomputation. The values represented as mean ± SEM. In all the analyses, *p* value of <
was taken as significant

Characterization of PAAm-g-Dxt

i) ^{1}H-NMR spectroscopy

Figure 02 shows the ^{1}H-NMR of native Dxt (A), AAm (B) and PAAm-g-Dxt (C). In case of native Dxt, signals were seen at 4-5, 3-4 and 2.5 ppm are due to 3 and 6-linked-*α*-D-glc*p*residues, protons in C2, C3, C4, C5, C6 and DMSO respectively which was used as solvent. In the case of AAm, the signals were at 5.6, 6.1, 7.2-7.6 and 2.5 ppm are related to CH_2, CH of Dxt, $CONH_2$ protons of AAm and DMSO respectively. But for PAAm-g-Dxt copolymer, apart from the signals of Dxt, new signals at 1.5, 2.2 and 7.0 ppm were observed due to CH_2 and CH and $CONH_2$ protons of PAAm respectively.[21] This corroborates the grafting reaction of PAAmon dextran.

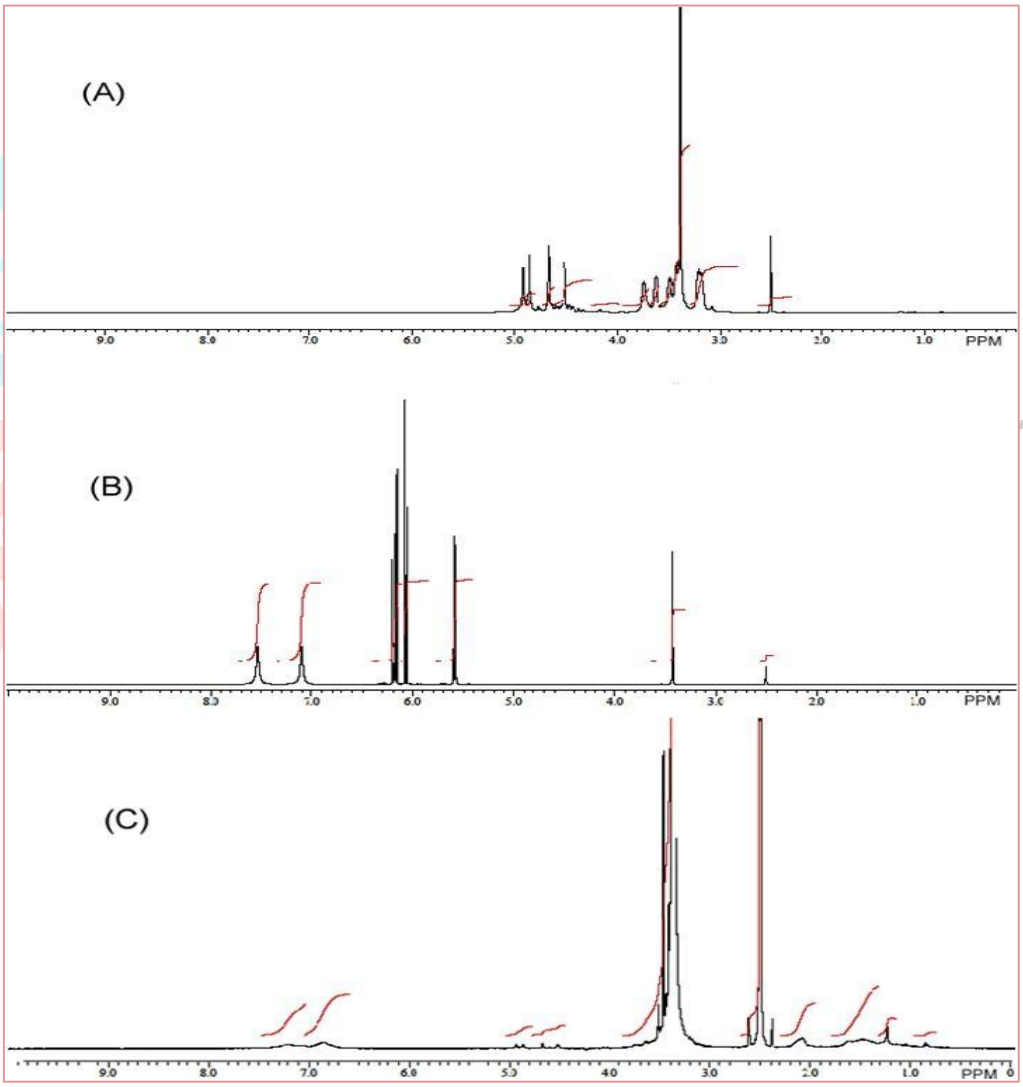

Figure 2 ^{1}H-NMR peaks of Dxt (A), AAm (B) and PAAm-g-Dxt (C)

ii) FTIR analysis

In case of dextran, bands were noticed at 3410, 1660, 1150, 1010, 2920 and 900 cm^{-1} due to -OH, carbonyl, alcoholic hydroxyl, cyclic aldehyde groups and α- glucopyranose ring respectively (Figure 03A). In case of PAAm-g-Dxt, the bands were noticed at 3450, 3220, 1640, 1450, 2950, 1150, 1010 and 900 cm^{-1} due to –OH, – NH, and primary amide aliphatic –CH, alcoholic OH groups and α-

glucopyranose ring respectively. So, this is the evidence for grafting reaction (Figure 03B). While in case of PAAm-g-Dxt, the peaks were noted at 3400, 2990, 1650, and 1410 cm^{-1} because of the –OH, aliphatic –CH, primary amide and COO^- groups respectively. However, the peak appeared at ~3220 cm^{-1} in the spectra of PAAm-g-Dxt is disappeared indicating the partial hydrolysis of grafted copolymer (Figure 03 C)[22]

iii) Elemental analysis

A 0.00%, 39.27% and 7.15% of nitrogen (N), carbon (C) and hydrogen (H) were noted with native Dxt. But the PAAm-g-Dxt has shown 14.22% N, 42.16% C and 9.59% H; the sizable increased 'N' may be attributed to the existence of $–CONH_2$ groups on Dxt after grafting. Whereas, in case of hydrolyzed PAAm-g-Dxt, weobserved 4.28% N, 28.72% C and 6.50% H the % nitrogen was declined to 4.28 in hydrolyzed copolymer that may be due to change of $–CONH_2$ to –COOH groups (Table 02). This endorses grafting and hydrolysis.[23]

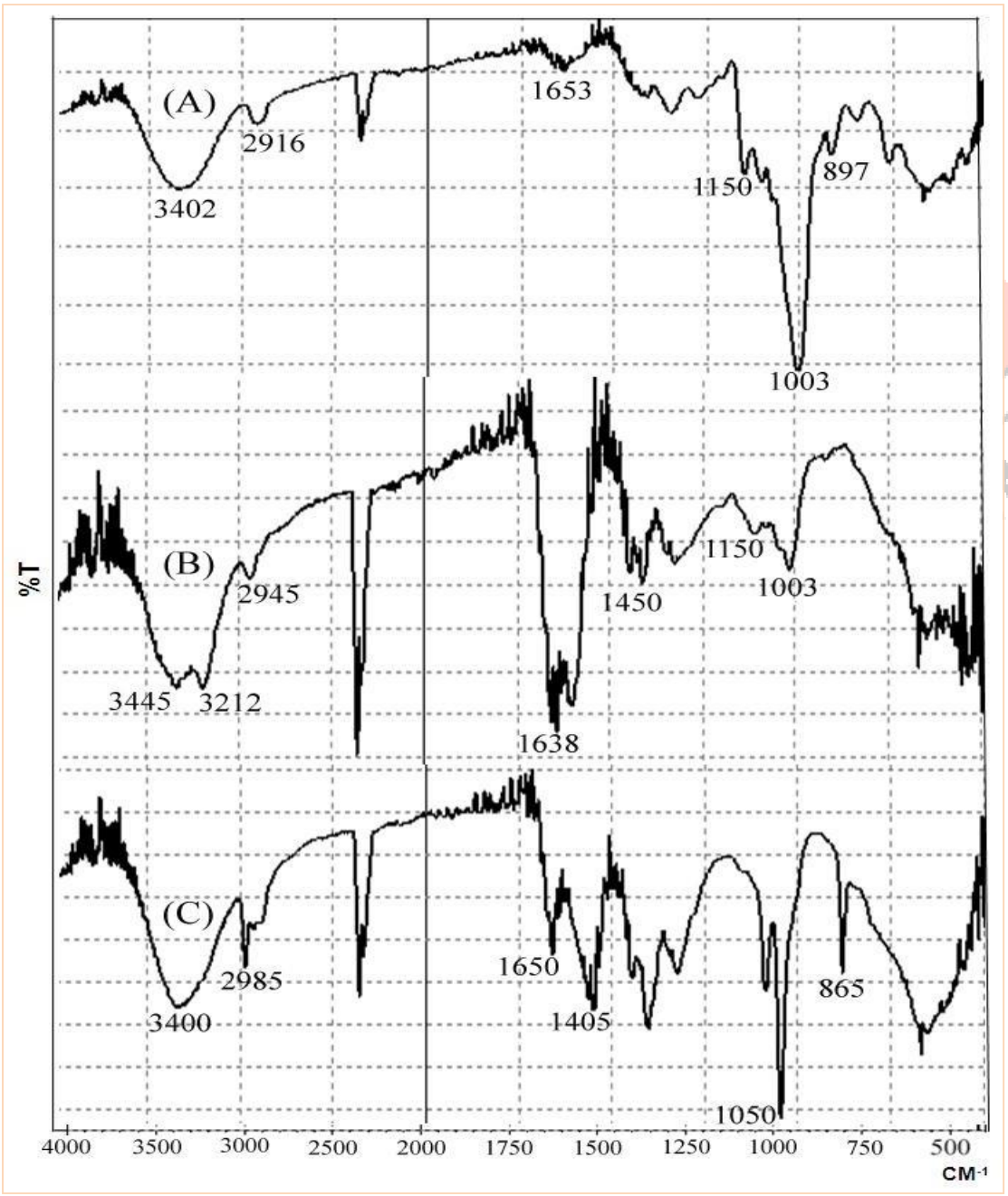

Figure 03 FTIR bands of Dxt (A), PAAm-g-Dxt (B) and hydrolyzed PAAm-g-Dxt (C)

iv) Determination of neutralization equivalent

Table 13 also shows the NE values for native Dxt, PAAm-g-Dxt and hydrolyzed PAAm-g-Dxt. During alkaline hydrolysis, the mol. weight of polymer will be unaltered, for the reason that one –OH group is added against the removal of one NH_3 group. Therefore, larger the number of $-COO^-$ groups, smaller will be the NE. The calculated NE values suggest that the hydrolyzed PAAm-g-Dxt has larger $-COO^-$ groups than the un-grafted Dxt. These $-COO^-$ functional groups are held responsible for electro-sensitivity of graft copolymer.

Table 02 Data of neutralization equivalent and elemental analysis

Polymer	NE	Elemental analysis		
		N (%)	C (%)	H (%)
DXT	1829.78	0.00	39.27	7.15
PAAm-g- Dxt	1920.40	14.22	42.16	9.59
Hydrolyzed PAAm-g- Dxt	426.17	4.28	28.72	6.50

v) Thermogravimetric analysis (TGA)

Figure 04 shows the TG curves for Dxt and PAAm-g-Dxt. In case of Dxt, the following thermal events were noted; up to 200 ^oC with 11.50% weight loss- loss of bound and unbound moisture from dextran. Up to 400 ^oC with 75.60% weight loss and up to 600 ^oC with 84.20% weight loss- decomposition of Dxt But, in case of PAAm-g-Dxt; up to 200 ^oC with 15.30% weight loss, up to 400 ^oC with 70.22 % weight loss and up to 600°C with 80.50% weight loss. There was a constant weight loss with PAAm-g-Dxt and percent residual mass was larger i.e., 19.25 % for of PAAm-g-Dxt as compared native Dxt i.e., 15.27%. This point towards the thermo-stability of PAAm-g-Dxt than the native Dxt

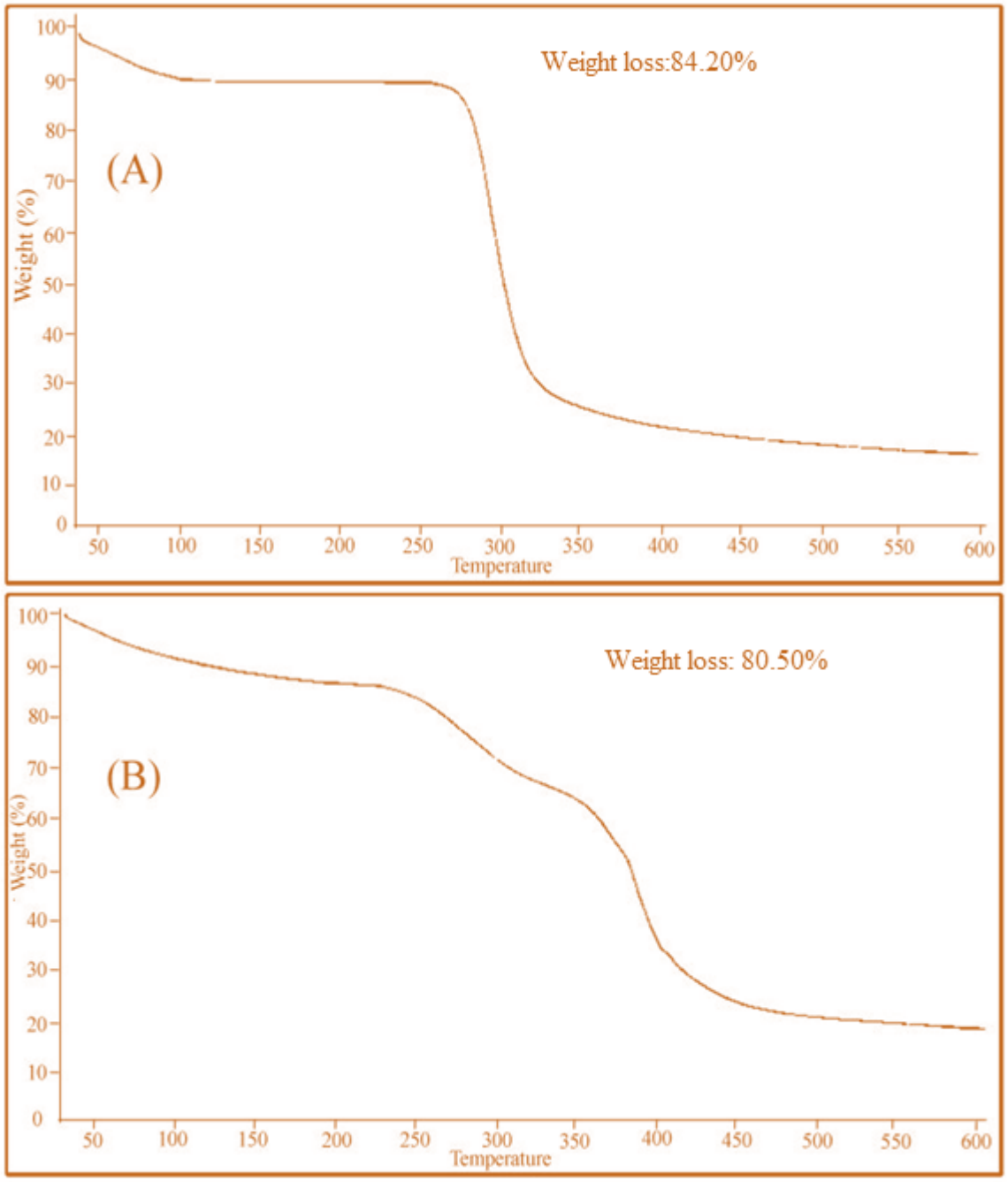

Figure 04 TGA analysis of Dxt (A) and PAAm-g- Dxt (B)

Evaluation of ETDDS

i) Hydrogel reservoir

The reservoir gels were translucent and their pH was between 6.21 and 7.01, which is in the pH range of skin; while drug content was in the range of 83.28 to99.51 % (Table 03).

ii) Rate controlling membranes

The RCMs were flexible and smooth. The RCMs thickness was found between 49 and 90µ; the thickness was increased with increase in GA. The RCMs found permeable to vapors through them. With increase in concentration of GA, there was a decrease in WVT rate (Figure 05). The tensile strengths of the RCMs reveal that as the concentration of GA was increased, the tensile strength was also increased (Table 04).

Table 03 Results of drug content, pH and thickness of RCM

Formulations	Reservoir gel		Thickness of RCM(µ)
	Drug content (%)	pH	
DXT1	97.35 ± 1.12	6.81	49 ± 0.98
DXT2	99.51 ± 1.21	6.22	50 ± 0.87
DXT3	83.28 ± 1.07	6.48	59 ± 0.79
DXT4	85.65 ± 1.27	6.98	60 ± 0.69
DXT5	85.03 ± 1.16	7.01	66 ± 1.05
DXT6	84.45 ± 1.49	6.89	90 ± 1.12
DXT7	90.42 ± 1.21	6.41	72 ± 0.93
DXT8	99.06 ± 1.27	6.21	79 ± 1.08

Table 04. Tensile strength and extension values of RCMs

RCM	Tensile strength (kg/cm^2)	Extension (mm)
Dxt1	0.206	31.96
Dxt6	0.288	32.24
Dxt7	0.414	6.12
Dxt8	0.616	1.95

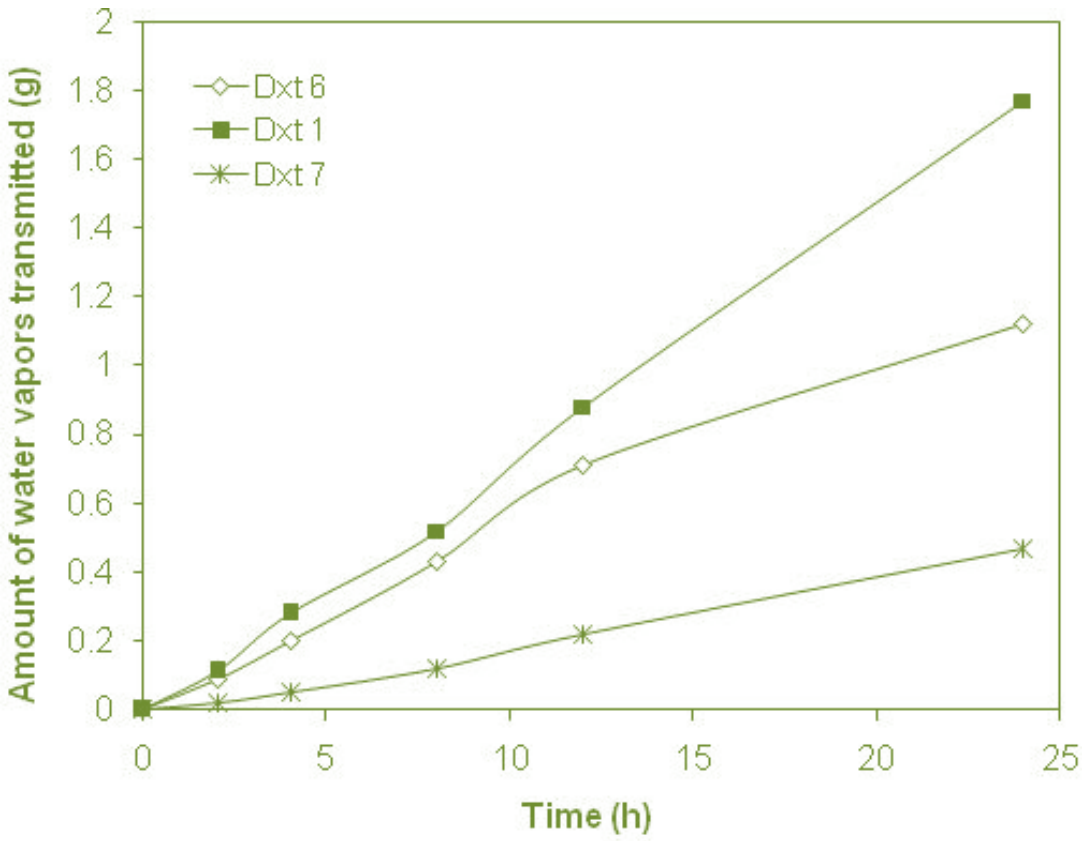

Figure 05 WVT profiles of RCMs

Scanning electron microscopy

The SEM study was done to assess the effect of crosslinking on the surface structure of RCMs. The SEM images in Figure 06 indicate that the RCM Dxt6 has confirmed rough and dense surface than the Dxt1 RCM. When the amount of GA increased, the RCMs might have undergone shrinkage resulting in rough and densesurface of membrane.

Differential scanning calorimetry

The results of DSC analysis of Dxt1 and Dxt6 RCMs are presented in Figure 07. The Dxt1 RCM has exhibited an endothermic peak at 103.81 ^{o}C. But the Dxt6 RCM has shown an endothermic peak at 115.5 ^{o}C. We noticed an increase in temperature of endothermic peak of Dxt6 RCM; this might be due to increasedtoughness of RCM due to increase in amount of GA

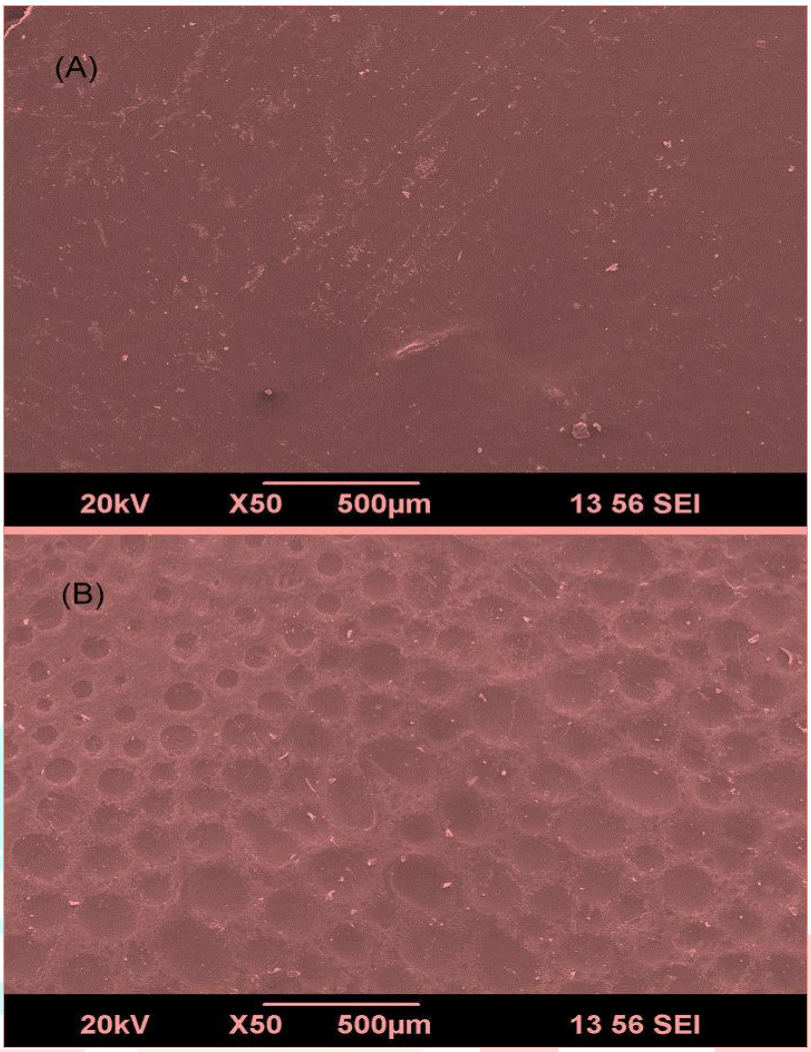

Figure 06. SEM images of Dxt1 (A) and Dxt6 RCMs (B)

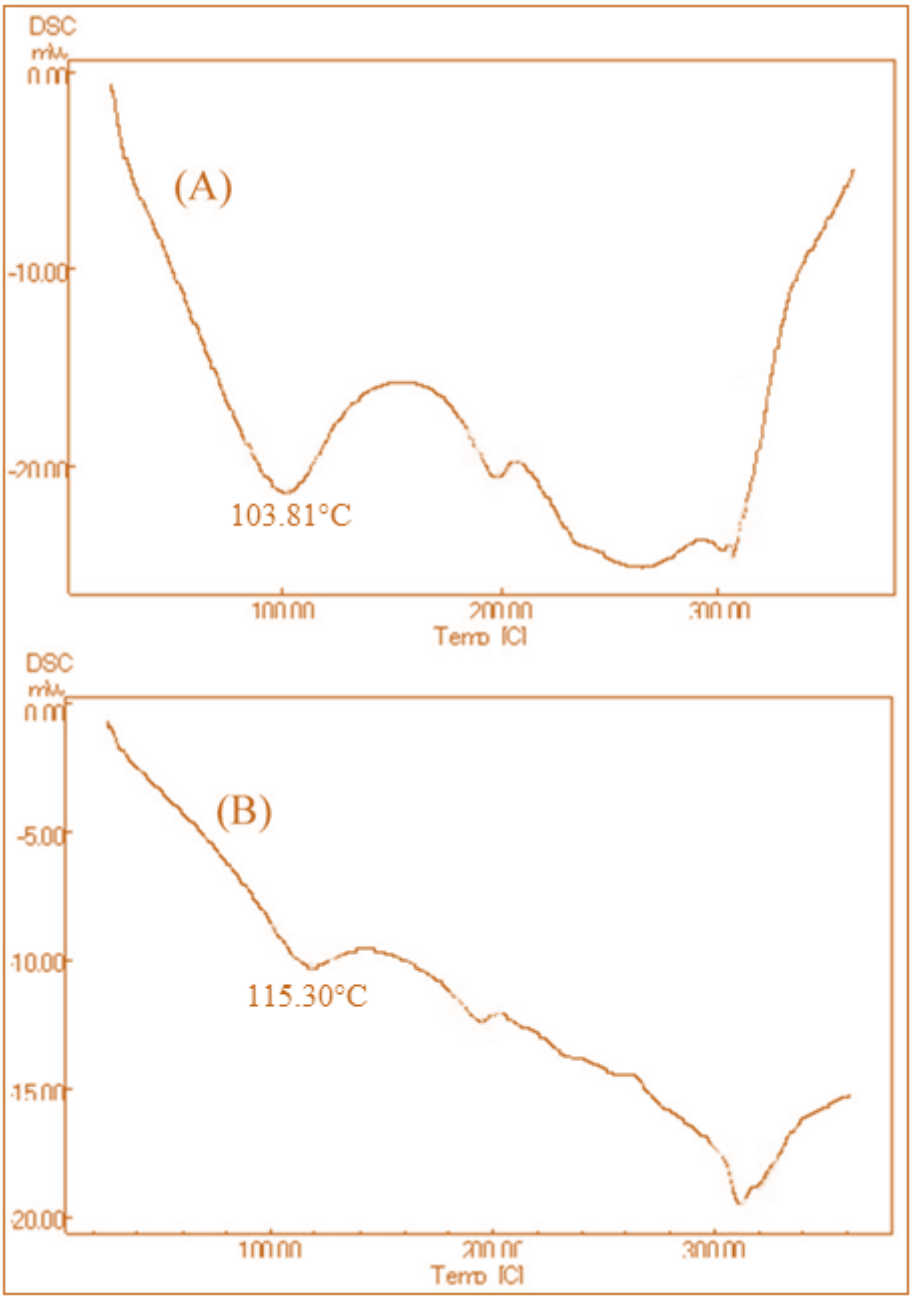

Figure 07. DSC analysis of Dxt1 (A) and Dxt6 (B) RCMs[103]

X-ray diffraction study

The results of X-ray analysis of Dxt1 and Dxt6 RCMs are given in Figure 25. The Dxt1 RCM has given away the typical intense peaks in the 2 range of 5º to 20º. Where, Dxt6 RCM has shown intense peaks between 2 of 5º and 30º. Nevertheless, the peak intensity for Dxt6 RCM is high when compared to Dxt1 RCM. It is due tohigher amount of GA in the membranes.

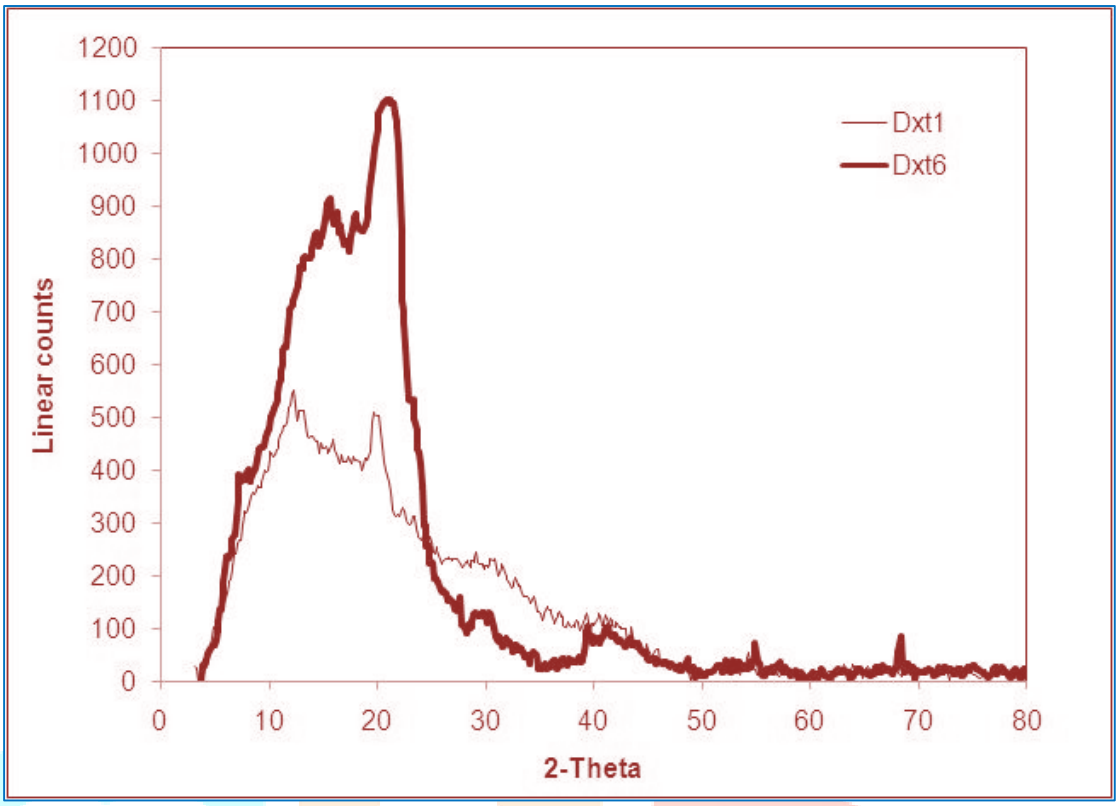

Figure 08. X-ray analysis of Dxt1 and Dxt6 RCMs[103]Drug permeation

The drug permeation results of ETDDS are depicted in Figures 09-12. The drug permeation was observed as slow in the absence of electric stimulus. The drug permeation enhancement was dependent upon the strength of supplied electric current.

An insignificant quantity of drug was permeated from ETDDS when electric stimulus was absent. A 70.35 % of drug permeation was recorded from Dxt1 formulation after 24 h. The drug permeation got reduced, when the concentration of PAAm-g-Dxt was increased in the formulation. It which may be due to fact that at higher amount of graft copolymer, there will be an increased viscosity of gel leading to reduced diffusion of drug. At the same time, drug release was reduced when the amount of GA was increased; this may be due to increased rigidness of RCMs which leads to condensed pore size resulting in decreased drug permeation (Figure 09A).

Whilst, the permeability rate of drug was increased when the electric stimulus of 2 mA was applied (Figure 09B) “The steady state fluxes were improved by 1.6 folds after application of electric current. A drug permeability of 90.20 % was witnessed from Dxt3 formulation after 24 h; this may be attributed to electrical sensitivity of the copolymer hydrogel with highest amount of PAAm-g-Dxt copolymer. A significant enhancement in drug permeation was noticed with applied electric current. The drug permeation tends to decrease with increased concentration of GA (Figure 09A). An

improvement in permeation rate was seen with increasing electric stimulus from 2 to 8 mA (Figure 10).

The calculated steady state flux (Jss) and the permeability coefficient (Kp) values are shown in Table 05 and Figure 11. The calculated enhancement factors (*EF*)were between 1.457 and 1.762. The flux values in the absence of electric stimulus were ranged from 0.0211 to 0.0743 mg/cm^2/h, but the flux values with electric stimulus were greater and ranged from 0.0630 to 0.0896 mg/cm^2/h (Figure 28).

For corroborate the electric responsiveness of ETDDS, the permeation study was also performed under "on–off" electric stimulus mode (Figure 12). With electric "ON" mode, higher permeation was seen and permeation got reduced when electric current was turned 'off.' The mechanism goes like; when electric stimulus was applied to the negatively charged PAAm-g-Dxt gel, the counter ion of polyion travels towards negative electrode, while polyion is static and other free ions of gel move towards the opposite electrodes across the gel. This creates difference in osmotic pressure across the hydrogel; this acts as driving force (either shrinking or swelling) for release of drug from the hydrogel.

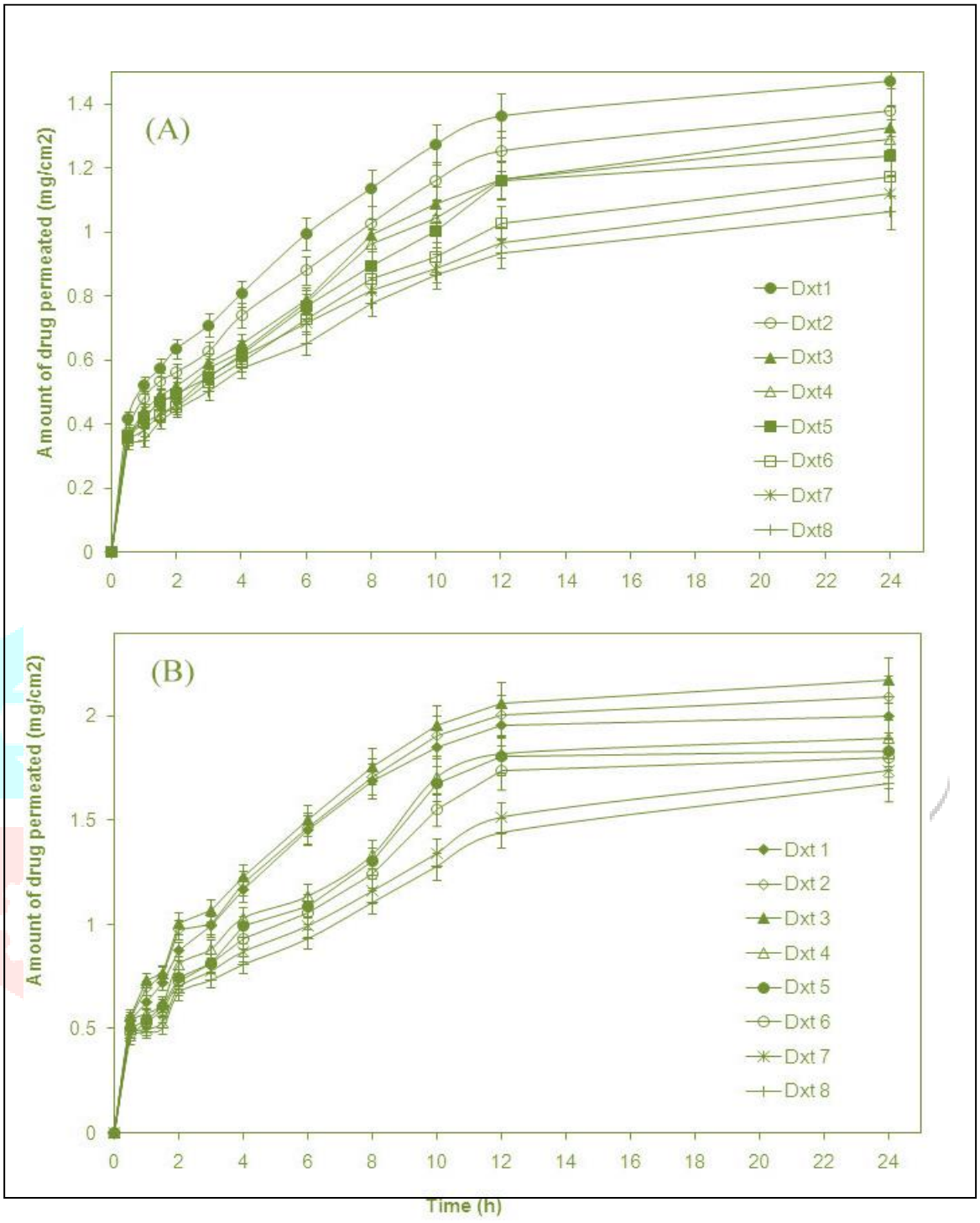

Fig. 09 *In-vitro* drug permeation without electric stimulus (A) and with electricstimulus (B)

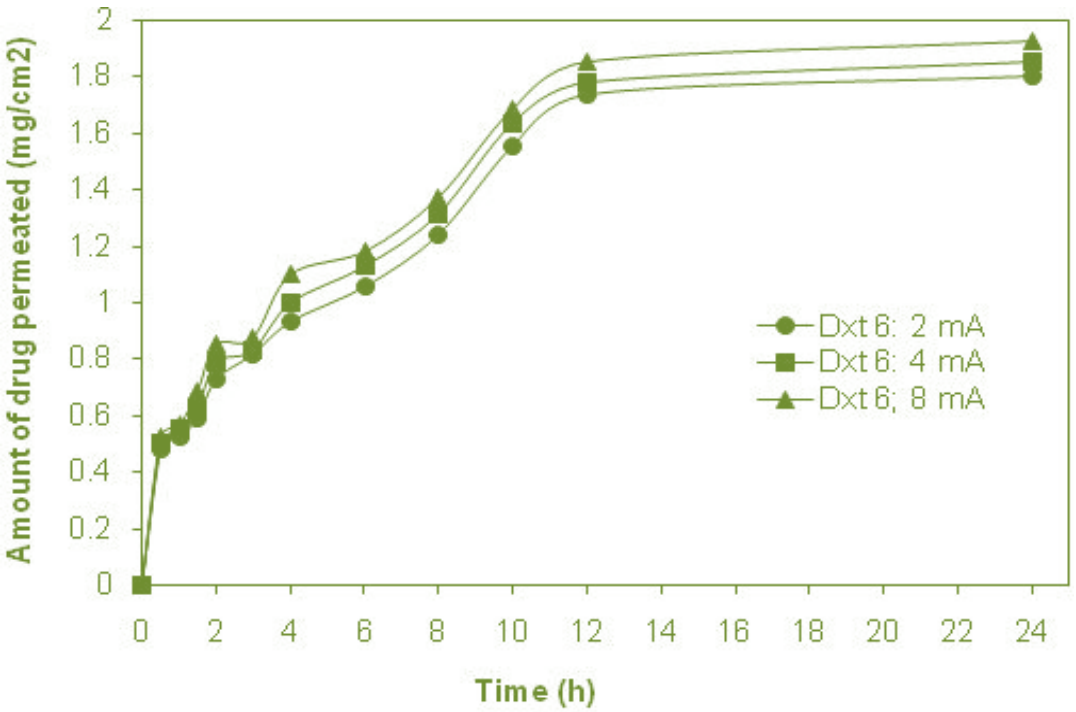

Figure 10 Drug permeation with varied electric current strengths

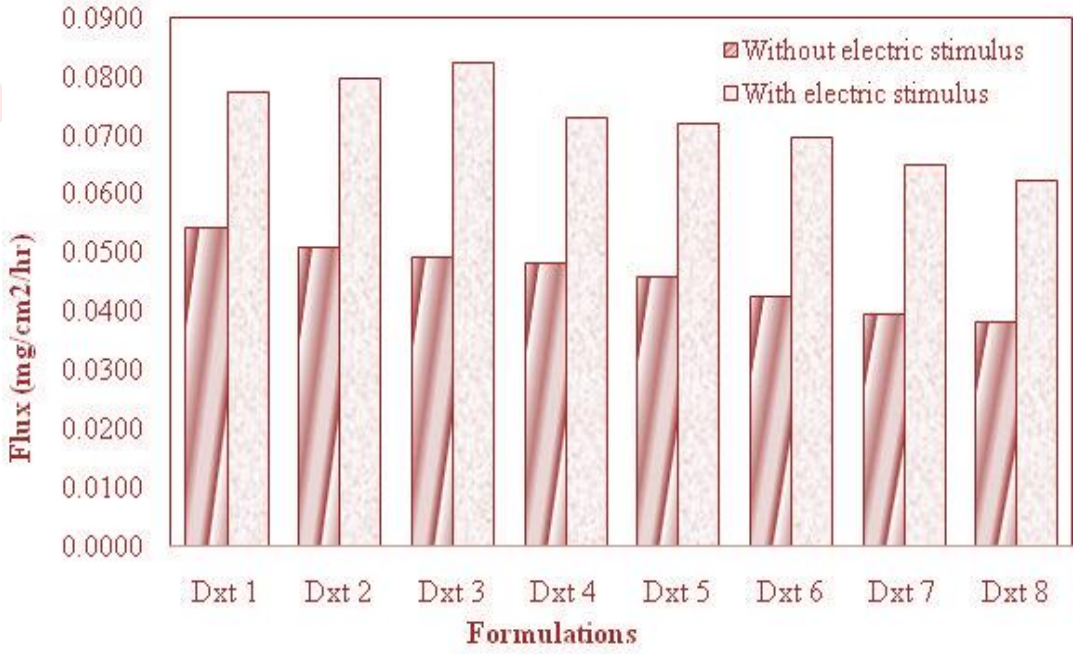

Figure 11. Flux values in the absence or presence of electric stimulus

Table 05 Date of flux, permeability coefficients, and enhancement factors

Formulations	Without electric stimulus		With electric stimulus		
	Jss ($mg/cm^2/h$)	Pm (mg/hour.cm)	Jss ($mg/cm^2/h$)	Pm (mg/hour.cm)	EF
DXT1	0.0743	0.0189	0.0694	0.0248	1.726
DXT2	0.0211	0.0140	0.0896	0.0253	1.457
DXT3	0 .0693	0.0176	0.0724	0.0270	1.762
DXT4	0.0684	0.0133	0.0630	0.0216	1.809
DXT5	0.0560	0.0157	0.0821	0.0224	1.655
DXT6	0.0327	0.0179	0.0799	0.0148	1.618
DXT7	0.0497	0.0181	0.0750	0.0266	1.585
DXT8	0.0582	0.0177	0.0824	0.0259	1.713

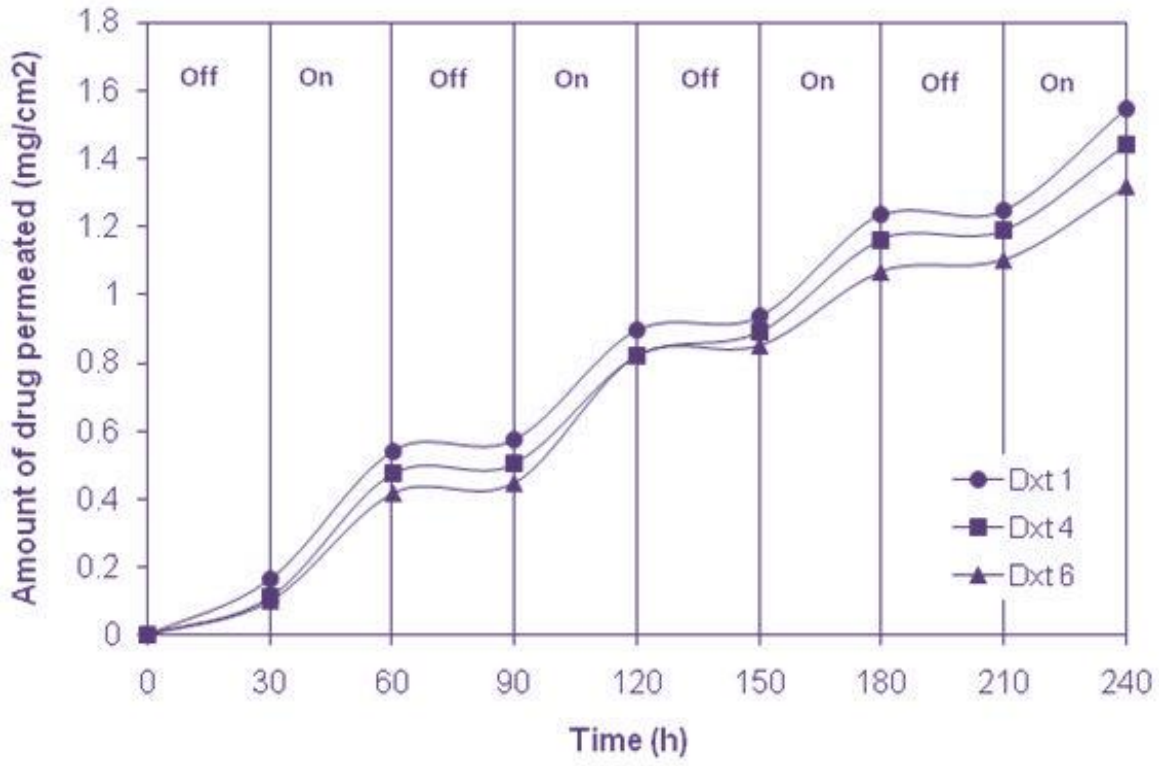

Figure 12. On-Off release behavior of ETDDS

Histopathological evaluation of skin

The Table 06 and Figure 13 depict the alterations of skin structure before and after electrical stimulation. It is evident from results that the stratum corneum of normal skin tends to be intact with preserved structural integrity, without inflammation or changes in the skin appendages, further there were no edema of sub- epidermal region and swelling of collagen fibers. But the skin which received electric stimulus shows slight looseness in the intactness of stratum corneum; the cell integrity was loosened with the edema of sub-epidermal region and swelling of collagen fibers was seen; there was a degeneration of skin appendages

Table 06 Scores of histopathology studies

Sl. No	Parameters	Normal skin	Skin treated with electric stimulus (2 mA)
01	Intactness of skin	1	2
02	Liquification of epidermis	0	2
03	Odema of Subepidermis	0	2
04	Swelling of collagen fibers	0	3
05	Inflammation	0	0
06	Erosion of skin appendages	0	2

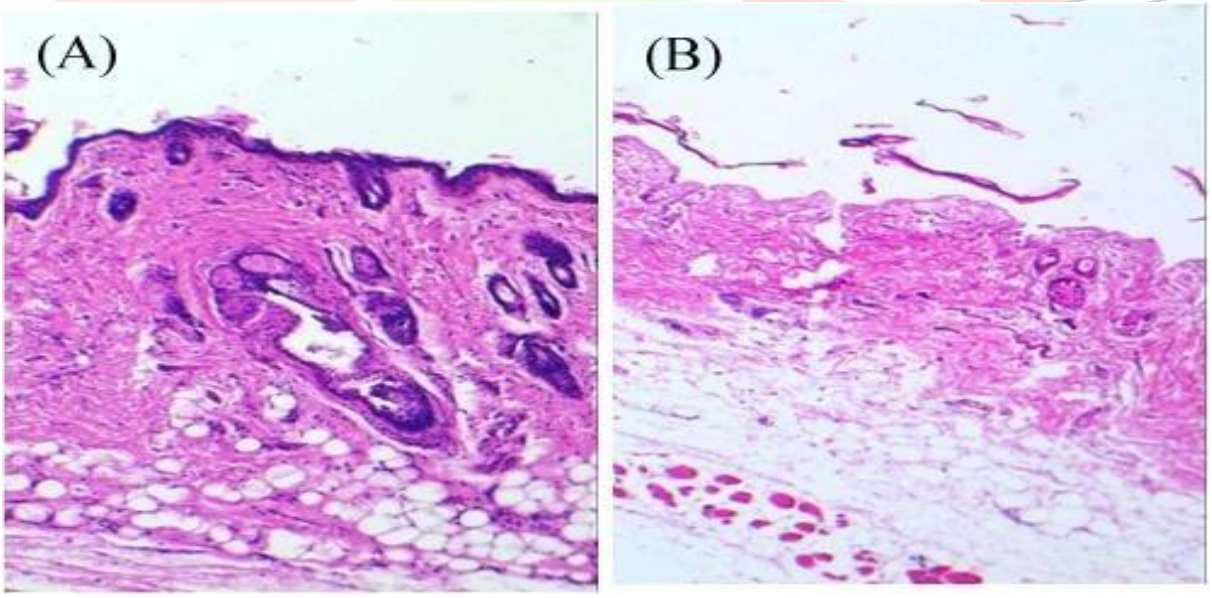

Figure 13. Histopathology images of rat skin before (A) and after electricalstimulation (B)

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