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FORMULATION AND EVALUATION OF HYALURONIC ACID NIMESULIDE GELS

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ABSTRACT
Hyaluronic acid is a polymer of disaccharide, themselves composed of D Glucuronic acid and D-N acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds. In the Present work an attempt is made to prepare HA gels containing Nimesulide. the gels prepared are subjected to extensive rheological evaluation, drug content estimation, pH measurement, stability study and drug release study across hairless albino skin rat. The influence of various permeation enhancers on rat skin permeation was studied. The major outcomes of the research are as follows. Gels prepared using HA revealed a pH of 6.45. Since HA is a component of the human skin no hypersensitivity reactions can be expected when used as a carrier in preparation of topical gels. During its shelf life, rheological evaluation studies indicated that there is no significant change in the viscosity of the formulation. The prepared HA gels had a greater initial rate of permeation as compared with the marketed formulations. From the results of the experiment. we can expect a superior onset of therapeutic action as compared with the marketed formulation. Since HA is an endogenous component involved in cellular signaling, wound repair, morphogenesis and matrix organization these gels do not cause skin irritation and hypersensitive reactions. The rate of skin permeation of the drug increased with increasing concentration of the permeation enhancer (PEGs) until a certain threshold value. At very high concentrations of the permeation enhancer, the rate of permeation has decreased suggesting intramolecular attraction of the PEGs thus limiting the rate of permeation.

KEYWORDS: Hyaluronic acid (HA), Rat skin Permeation , Poly ethylene glycol (PEG), HA gels, Rheological studies.

INTRODUCTION
Hyaluronic acid (HA) or hyaluronan is a linear polysaccharide that consists of alternating units of a repeating disaccharide, β -1,4- D -glucuronic acid– β -1,3- *N* -acetyl- D –glucosamine**(Figure-1)**. HA is anon-sulfated glycosaminoglycan and is found throughout thebody, from the vitreous of the eye to the extracellular matrix(ECM) of cartilage tissues.[1] HA is an essentialcomponent of the ECM, in which its structural and biologicalproperties mediate its activity in cellular signaling, woundrepair, morphogenesis, and matrix organization.[2,3] Additionally, HA is rapidly turned over in the body by hyaluronidase, with tissue half-lives ranging from hours to days.[4] HA anditsderivatives have been clinically used as medical products for over three decades.[5] More recently HA has become recognized as an important building block for the creation of new biomaterials with utility in tissue engineering and regenerative medicine.[6–9] HA can be modified in many ways to alter the properties of the resulting materials, including modifications leading to hydrophobicity and biological activity.[10] Chemical modifications of HA have been extensively reviewed.[11] These HA derivatives fall into two primary categories: "monolithic" and "living".[12] In most cases, living HA derivatives are required for clinical and preclinical uses in 3D cell cultures and in vivo cell delivery.[13]

Due to their excellent biocompatibility, non-toxic nature, and tenability of properties and degradation, HA hydrogels are potentially useful for molecule delivery applications. Additionally, photo polymerization provides a simple technique for the encapsulation of molecules for delivery applications.[14, 15] The delivery of proteins from glycidyl methacrylate derivative of HA (GMHA), alone and combined with poly(ethylene glycol), was investigated with a range of hydrogel formulations.[16,17,18] Gene therapy approaches are becoming useful in regenerative medicine to alter the gene expression of cells towards directed tissue repair, thus the local and controlled delivery of DNA is important. In this work, the release profiles were dependent on the extent of cross-linking and amount of HA incorporated and the activity of the released DNA was dependent on the encapsulation conditions and material formulations. Likewise, Shea and co-workers

investigated vector delivery from hydrogels with acrylated HA and 4-arm poly(ethylene glycol) precursors and assessed delivery profiles based on material compositions.[19] This provides a non-viral approach for the local delivery of DNA and illustrates the importance of release on hydrogel properties and the specific vector used.

Skin penetration enhancers (PE) are used to remove the barrier resistance of the stratum corneum reversibly. PE allows drugs to penetrate to the viable tissues and thus enter the systemic circulation.[20] The rate of permeation of the drug molecule through topical route is increased by using chemical agents or physical method.[21,22] The use of permeation enhancers like Propylene glycol, low molecular weight poly ethylene glycols including ethanol has been documented elsewhere.

The present study involved the formulation and evaluation of HA gels containing Nimesulide and the influence of various permeation enhancers on rat skin permeation. Permeation enhancers such as propylene glycol, PEG 400, PEG 600, were taken at various concentrations. We conducted solubility studies for Nimesulide on PEG 4000, PEG 6000 during which we found solubility enhancement. Therefore we incorporated PEG 4000 and PEG 6000 in the gel to study the permeation. Apart from rat skin permeation studies, parameters such as rheological behavior, stability.

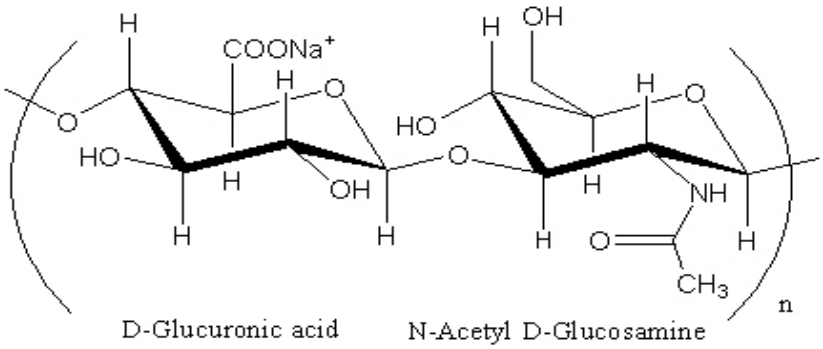

Figure 1: Schematic illustration of the principal structure of HA.

MATERIALS AND METHODS

Solvents and Chemicals

Nimesulide (purity 99.60 % w/w) was obtained as a gift sample from Ajanta Pharma Ltd. Ethanol, Sodium Hydroxide, and Potassium dihydrogen phosphate were purchased from Merck Ltd (Mumbai, India). Hyaluronic acid is purchased from M/s Kumar Organic products Ltd, Bangalore. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Propylene glycol, PEG 400, PEG 600, PEG 4000, PEG 6000 and Sodium methyl paraben were purchased from S.D Fine chem. Ltd (Mumbai, India). All other chemicals and reagents were of analytical grade.

Instrumentation

Double beam UV-Visble Spectrophotometer (Lab-India Analytical Instruments, India) synchronized to a computer work station using UV-Win software, magnetic stirrers (REMI Instruments, India), Brookfield Viscometer were used in the present study. The detector is set at a wavelength of 395 nm.

Preparation of HA hydrogels

A quantity of HA, permeation enhancer and glycerine were added as specified in **Table – 1**and mixed in about 15 ml of deionized water and mixed using a magnetic stirrer at room temperature for 30 minutes to obtain a homogenous gel. Nimesulide was then dissolved in 10 ml of ethanol. This ethanolic solution of the drug was then added to the prepared gel and mixed using a magnetic stirrer until homogeneity is achieved. Stirring speed was adjusted to minimize the air entrapment in the gel and deionized water was added to adjust the weight of the gel to 20 grams. The final gel formulation contained Nimesulide equivalent to 1 % w/w of Nimesulide. The prepared gel is then transferred into 50 ml polypropylene centrifuge tubes and preserved at 24^{o}C and 60 % Relative humidity until further use.

Evaluation of gels

The Nimesulide gels were subjected to extensive rheological evaluation, drug content estimation, pH measurement, stability study and drug release study across hairless albino skin rat.

pH measurement: The pH measurement was carried out by using a calibrated digital type pH meter by dipping the glass electrode and the reference electrode completely into the gel system so as to cover the electrodes.

Table 1: Composition of the HA gels prepared using various permeation enhancers.

	Weight in mg for 20 grams of the gel							
Sr. No	**HA**	**PG**	**PEG 600**	**PEG 400**	**PEG 4000**	**PEG 6000**	**Glycerin**	**equivalent to dissolved in 1 ml of EtOH**
F1	250	250	-	-	-	-	0.5 ml	20
F2	250	500	-	-	-	-	0.5 ml	20
F3	250	750	-	-	-	-	0.5 ml	20
F4	250	-	250	-	-	-	0.5 ml	20
F5	250	-	500	-	-	-	0.5 ml	20
F6	250	-	750	-	-	-	0.5 ml	20
F7	250	-	-	250		-	0.5 ml	20
F8	250	-	-	500	-	-	0.5 ml	20
F9	250	-	-	750	-	-	0.5 ml	20
F10	250	-	-	-	250	-	0.5 ml	20
F11	250	-	-	-	500	-	0.5 ml	20
F12	250	-	-	-	750	-	0.5 ml	20
F13	250	-	-	-	-	250	0.5 ml	20
F14	250	-	-	-	-	500	0.5 ml	20
F15	250	-	-	-	-	750	0.5 ml	20
MKT FORM	-	-	-	-	-	-	-	-

Drug content estimation

An accurately weighed 1 gm quantity of the gel was transferred into a 250ml stoppered volumetric flask and shaken vigorously with 2x25 ml quantity of methanol to extract the drug. The contents were filtered into a 50 ml volumetric flask and volume was made up to the mark with methanol. From the above solution, 0.5 ml was pipetted in to a 25 ml volumetric flask and volume was made up to 25 ml with methanol. The solution was estimated for the amount of Nimesulide.

***In-vitro* release studies**

The albino rat skin is obtained from the sacrificed animal at the pharmacology department in the college. Neither animal procurement nor sacrificing is performed as part of this experimental study. The skin was soaked in 0.32 N ammonium hydroxide solution[23] for 30 to 35 minutes to remove subcutaneous fat and hair. The skin was rinsed well with saline followed by distilled water. A 25ml Franz diffusion cell with a provision of water circulating jacket to maintain temperature was used for permeability study: 1 g of the gel was uniformly spread over the rat skin membrane and tied over the donor compartment. The skin was placed with stratum corneum facing the donor compartment and the dermis facing the receptor compartment containing 25ml phosphate buffer of pH 6.8. The receptor medium was magnetically stirred for uniform distribution and was maintained at a temperature of 37°C ± 0.2°C. 2 ml of sample was withdrawn from the receptor and replaced with fresh 2 ml phosphate buffer pH 6.8. The 2 ml withdrawn sample was made up to 25 ml with phosphate buffer pH 6.8 and the sample was estimated using RP-HPLC.

Rheological studies

Brookfield viscometerwas used for the studies. Rheological observations were studied at HA concentrations ranging from 0.5 % - 2.0 % w/w. 1.0 g of the gel containing HA at 1.25 %w/w was taken and dipped into the gel till the notch on the spindle touched the gel surface. This spindle was rotated at 5.0 rpm and dial reading was recorded until 2 consecutive similar readings were obtained. Similarly dial readings were recorded at 10.0, 20.0, 40.0, 50.0 and up to 100 rpm. As soon the sample was sheared at the highest rate, another set of dial readings were recorded by reducing the spindle rotation in the decreasing order to pool the data on the down curve**.** Rheograms were constructed by plotting the dial readings on the X-axis and rpm values along the Y-axis.

Stability study

Stability studies for Nimesulide sodium gels were carried out as per ICH guidelines.[24] The gel samples were stored at 25^{o}C, 60% relative humidity (RH) and 40^{o}C, 70% RH in stability chambers for a period of 6 months, samples were drawn at regular interval for stability analysis. At the end of 6 months assay was carried out to find out if there is any interaction between the drug and other ingredients of the formulation upon storage.

RESULTS AND DISCUSSIONS

Rheological observations

A graphical representation of the shear viscosity versus shear rate at 37^{o}C (human body temperature) for solutions of HA at different concentrations was shown in **Figure -3**. From the graph, it is clearly indicating that Newtonian behavior is visible at low shear rates followed by shear thinning at higher shear. These observations are co-incident with the results reported by Atoosa Malieki

et al[25] The cause of breakdown of the network junctions is attributed to shear thinning at higher shear. This type of behavior is also reported in previous studies on HA.[26-29] A strong rise in the η_{rel}($\eta_{rel} = \eta_{sample}/ \eta_{water}$) with increasing HA concentrations is observed at 40°C (**Figure -4)**. The samples were re-analyzed after cooling to 25°C. There is no apparent change in the η_{rel} due to temperature. This suggests that as the polymer concentration increases, the growth of entanglements creates a strong network that is not substantially deformed or reorganized. Rheological studies on stability samples at 40°C and 70% RH also indicated no significant change in the viscosity of the formulation.

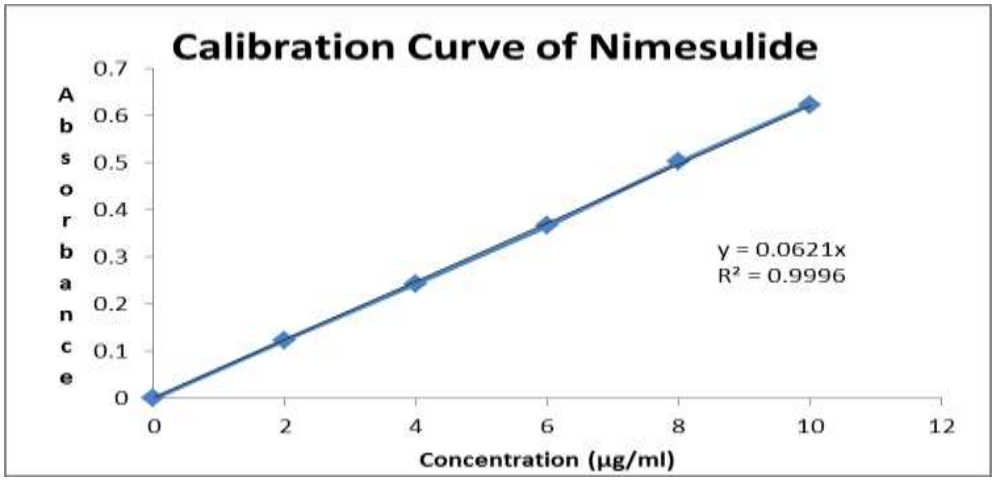

Figure 2: Calibration Curve of Nimesulide.

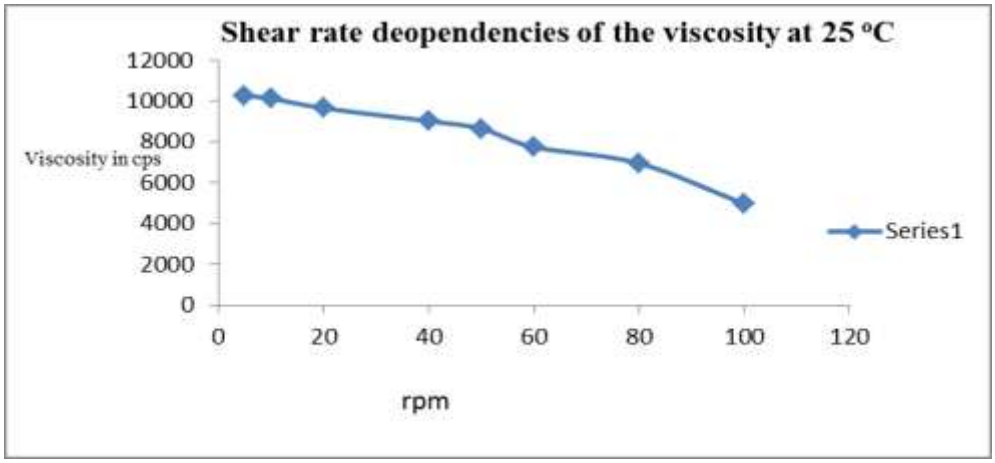

Figure – 3: Shear rate dependency of the viscosity at 25°C.

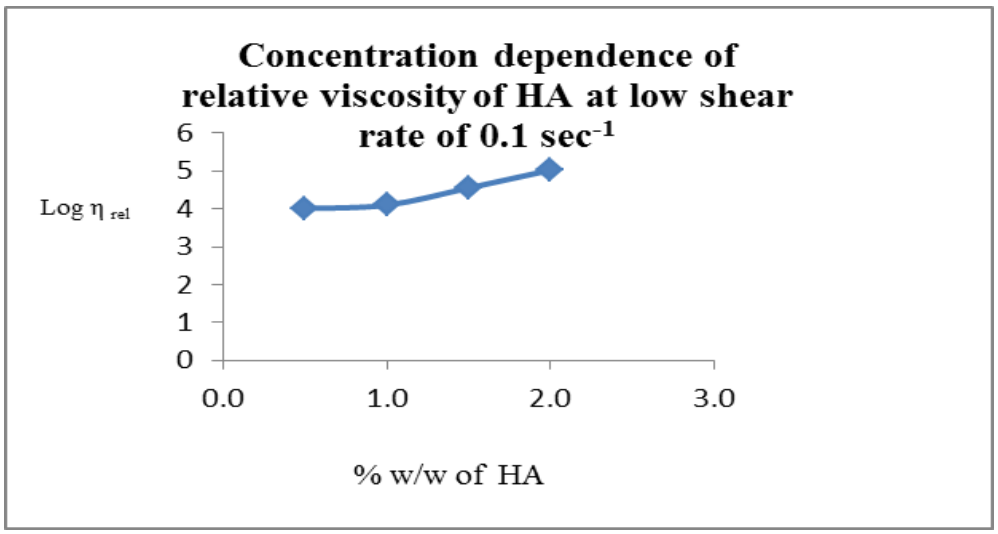

Figure – 4: Concentration dependence of the relative viscosity at a low shear rate for HA.

pH measurements

Gels prepared using HA revealed a pH of 6.45. The gels which have a pH value in the range of 5.5 to 7.5 are said to be most ideal, as they near the pH of the skin and do not cause irritation. Swamy et al[30] have reported pH values of 7.48 and 7.46 respectively for Nimesulide sodium gels made of Sodium carboxy methyl hydroxy propyl guar and hydroxy propyl methyl cellulose respectively. Our HA gel formulation reported a slightly acidic pH value. This may be attributed to the acidity of ethanol used as a humectant in our formulation.

Drug content estimation

Drug content of the HA gels was estimated by RP-HPLC method. Under the set parameters, the retention time of Nimesulide is 6.68 ± 0.1 minutes. Although the peak exhibited an asymmetry factor of 0.92, the method is adapted for the estimation of Nimesulide in HA gels. Any modification of the chromatographic parameters resulted in slight interference of the gel constituents with the Nimesulide peak. The number of theoretical plates were 10650 ± 185 indicating good efficiency of separation and detection. The run time of analysis is 8.0 minutes. None of the constituents of the gel interfered with the analysis of the Nimesulide.

In-vitro release studies

Permeation studies of the drug across hairless albino rat skin with HA based gels were conducted. The plot of % Drug retained in the gel versus time has been plotted (Figure – 5a to 5e). A lot of data can be inferred from the permeation studies. From all the graphical representations, it is inferred that the drug release pattern in the marketed formulation is almost zero-order kinetics throughout the permeation study. In contrast, HA gels demonstrated a biphasic pattern of penetration. During the 1st hour all the HA gels demonstrated a 1st order release after which the release followed a zero order process. An evaluation of the regression values (r^2), as tabulated in **Table 2** indicates that the overall process is zero order.

Compared to the marketed formulation that permeated about 10 % during the 1st one hour, the HA gels had a greater permeation effect. After the first 1 hour, HA gels containing propylene glycol at 1.25 % w/w demonstrated 55 % permeation. Keeping the percentage of permeation enhancer constant at 1.25% w/w, PEG 400 and PEG 600 have demonstrated 20.62 % and 51.49% drug permeation respectively indicating a possible superior onset of therapeutic action with HA gels. For PEG 4000 and PEG 6000 the drug permeation levels are 75.32 % and 40.72 % respectively. The effect of average molecular weight of PEG on the drug permeation during the 1st one hour is given in **Table – 3**. From table-2, the following points can be inferred; (a) Except for PEG 6000, the % drug permeation in-vitro increased with increased average molecular weight of the PEG. Low molecular weight PEG's which exist in the liquid form has maximal interactions while the higher PEG's tend to solidify themselves. Therefore high molecular weight PEG such as PEG 6000 may have formed a barrier layer inhibiting the drug permeation across the skin. (b) Except PEG 400, all the other PEG's used in the study demonstrated a small decline in the % drug permeation at the median concentration (2.5 % w/w) level of the permeation enhancer; however at higher concentration (3.75 % w/w) level the % drug permeation reached a maximal value. This anomalous behavior of PEGs has not been described in previous literature.

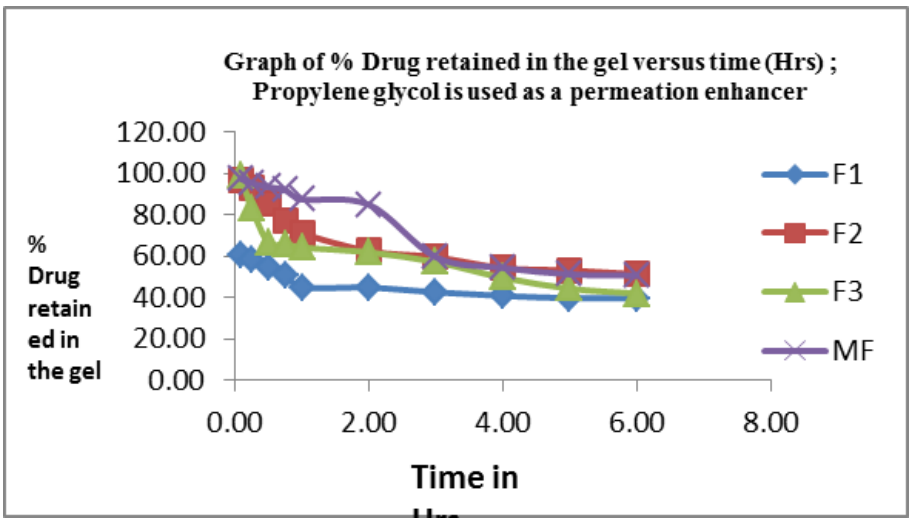

Figure – 5a: Schematic illustration of % Drug retained in the gel versus time in Rat skin permeation study *In-vitro* with propylene glycol as permeation enhancer.

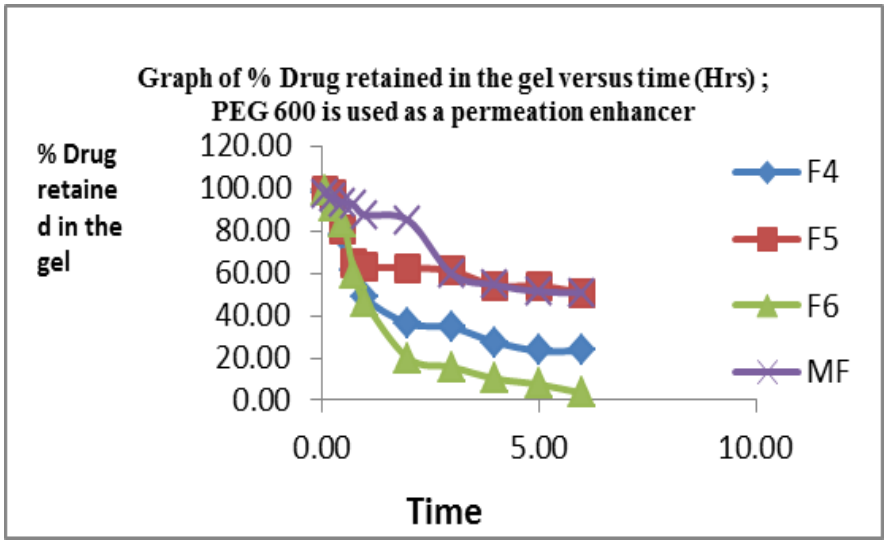

Figure – 5b: Schematic illustration of % Drug retained in the gel versus time in Rat skin permeation study *In-vitro* with PEG 600 as permeation enhancer.

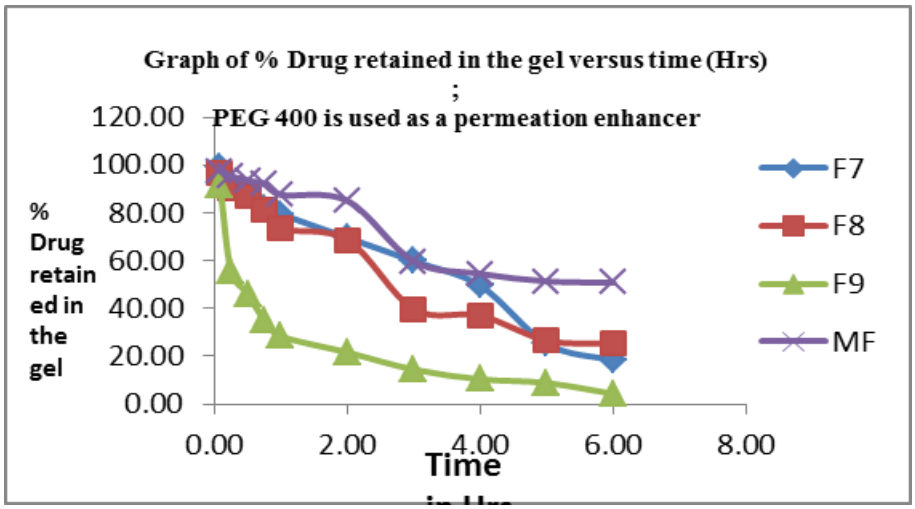

Figure – 5c: Schematic illustration of % Drug retained in the gel versus time in Rat skin permeation study *In-vitro* with PEG 400 as permeation enhancer.

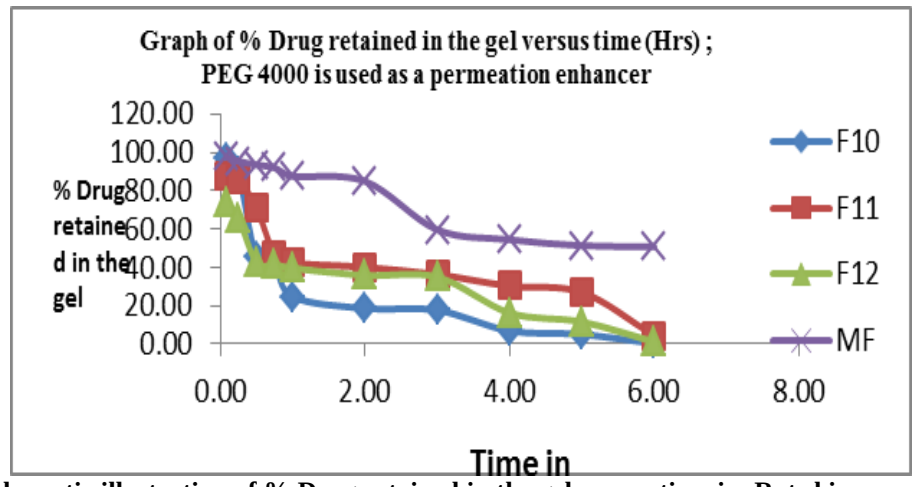

Figure – 5d: Schematic illustration of % Drug retained in the gel versus time in Rat skin permeation study *In-vitro* with PEG 4000 as permeation enhancer.

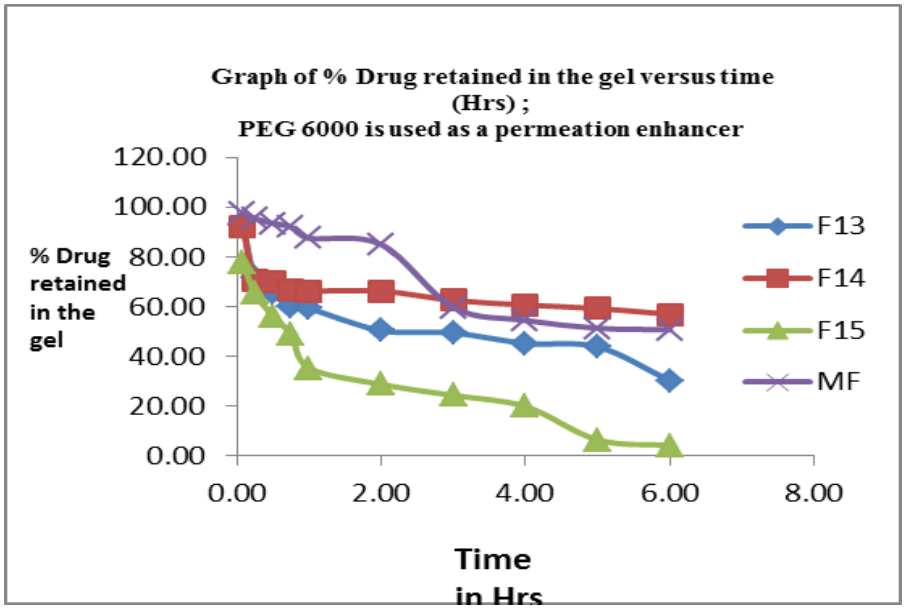

Figure – 5e: Schematic illustration of % Drug retained in the gel versus time in Rat skin permeation study *In-vitro* with PEG 6000 as permeation enhancer.

Table 2: Values for coefficient of regression (r^2).

Sr. No	Regression values for % drug release versus time for overall duration	Regression values for log % drug release versus time for overall duration
F1	0.740	0.701
F2	0.817	0.588
F3	0.746	0.338
F4	0.744	0.474
F5	0.631	0.397
F6	0.787	0.502
F7	0.982	0.656
F8	0.944	0.746
F9	0.654	0.376
F0	0.651	0.383
F11	0.778	0.592
F12	0.844	0.692
F13	0.759	0.466
F14	0.539	0.335
F15	0.855	0.755
MKT FORM	0.929	0.843

Table 3: Influence of PEG molecular weight on the permeation behavior across rat skin.

% Drug Permeated during 1st One hour			
	Concentration of the permeation enhancer		
Average Mol. Wt of PEG	**1.25 % w/w**	**2.5 % w/w**	**3.75 % w/w**
400	20.62	26.69	28.35
600	51.49	36.98	53.47
4000	75.32	57.56	60.36
6000	40.72	33.71	64.67

CONCLUSIONS

HA gels of Nimesulide containing various permeation enhancers were prepared and evaluated *In-vitro* using rat skin permeation studies. Quality control aspects including pH, stability studies, rheological behavior and drug content estimation were evaluated. The findings of

the present study were (a) At low concentration of HA, the gels exhibit a Newtonian behavior at low shear and gradually tend to undergo shear thinning (b) With increasing concentration of HA, there is a remarked increase in the viscosity of the gel at 40^{o}C; the apparent relative viscosity did not change significantly when similar measurements were taken at 25^{o}C. (c) The rate of drug permeation increases with increase in the molecular weight of the PEG up to a threshold level after which a decrease in the rate of permeation occurs which may be due to the solidification of the high molecular weight PEG's at body temperature. (d) The overall rate of drug permeation across rat skin followed a zero order process (e) since the initial rate of permeation is higher, a superior onset of therapeutic action can be expected as compared with the marketed formulation. (f) Since HA is an endogenous component involved in cellular signaling, wound repair, morphogenesis and matrix organization[2, 3] gels made of HA do not cause skin irritation and hypersensitive reactions. As compared with the traditionally used synthetic gel forming vehicles HA gels can offer a superior onset of therapeutic action. Since literature also suggested the absence of hypersensitivity reactions, future research must investigate the possibility of HA gels to cause skin irritations & hypersensitivity reactions. The compatibility of various other excipients with HA gels must also be investigated. It is our strong belief that HA can be substituted in place of other gel forming agents for topical applications so as to bring novel biocompatible formulations to the future generations.

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List of abbreviations

HA- Hyaluronic acid.
ECM- Extracellular matrix.
GMHA- Glycidyl methacrylate derivative of HA.
PE- penetration enhancers.
PEG- Poly ethylene glycol.
PG- Propylene glycol.
DNA- Deoxyribonucleic acid