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Research Article

REGULATION OF INFLAMMATION VIA ASA LOADED PROTEIN NANOPARTICLES

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ABSTRACT

The axiom of the current research was to formulate a protein based nanoparticles loaded with a NSAID to regulate chronic deformity which occurs due to inflammation as in case of rheumatoid arthritis, diabetic retinopathy etc. Two step desolvation method was espousedto prepare the formulation with gelatin; being biodegradable and non-toxic material as drug delivery vehicle and the drug of choice; aspirin which shows efficient results in the suppression of rheumatoid arthritis and diabetes induced eye inflammation. Effect of various experimental variables like temperature, stirring speed and concentration of cross-linking agent (glutaraldehyde) were observed. The entrapment efficiency of nanoparticles containing varied concentration of cross linker (glutaraldehyde) shows entrapment of drug in range of 51.74 -56.53% and the particle size was found in the range of 192 nm to 402 nm.In vitro release profile was applied on various kinetic models in order of zero order, first order, Higuchi equation and Korsmeyer-Peppas equation. Formulation-II treated reduced carrageenan induced paw edema by 35.29% and Formulation-I treated reduced the inflammation by 31.37% as compared to control in non-diabetic rats. Ex vivo study on goat eyes exposed satisfactory result of drug penetration and retention at corneal tissues. The Formulation was stable for all parameters after 60 days.

Keywords: Gelatin, aspirin, anti-inflammatory activity, paw edema, corneal retention.

INTRODUCTION

Inflammation (Latin, inflammare, to set on fire) is characterized by the compound bioticreaction of vascular tissues to destructive impetuses, such as pathogens, impaired cells, or aggravations[1]. Inflammation is not a replacement for infection. Inflammation is categorized by amplified leukotrienes and prostaglandin echelons.

Preliminary information indicates that there is an elevation of at least one specific prostaglandin, i.e. prostaglandin F2α, in the serum of the diabetic persevering who has diabetic retinopathy[2]. Ocular tissues made known to produce prostaglandins embrace iris, ciliary body, conjunctiva, cornea[3] and retina, while prostaglandin production by corneal epithelial, endothelial and trabecular meshwork cells have been considered under tissue culture conditions[4]. Arthritis is a general term that designates inflammation in joints. Rheumatoid arthritis is a type of chronic (ongoing) arthritis (resulting in pain and swelling) that occurs generally in joints on both sides of the body (such as hands, wrists, and knees).

Aspirin is part of a group of medications called nonsteroidal anti-inflammatory drugs (NSAIDs), but differs from most other NSAIDs in the mechanism of action. Though it and others in its group called the salicylates, have similar effects (antipyretic, anti-inflammatory, analgesic) to the other NSAIDs and constrain the same enzyme cyclooxygenase, aspirin (but not the other salicylates) does so in an irreversible manner and, unlike others, affects more the

COX-1 variant than the COX-2 variant of the enzyme[5]. There has been tremendous progress within the last 30 years in the field of nanotechnology, concerning nano devices apposite for drug delivery, from the first liposomal approach[6] to innumerable contemporaneous colloidal systems like nanoparticles, dendrimers, CNTs etc. that empower temporal and spatial site-specific delivery. Compared to other colloidal carriers like liposomes, biopolymers epitomize superior stability when in connection with biological fluids and their polymeric nature countenances one to obtain controlled and sustained release of the entrapped drug.

Among the accessible potential colloidal drug carrier systems casing the size range described, protein- based nanoparticles play an imperative role[7]. Most often, serum albumin obtained from human, bovine, legumin, etc. as well as gelatin was used as the initial material for the preparations.While gelatin and the delivery systems based on this polymer are biocompatible and biodegradable without toxic degradation products[8, 9, 10, 11].

The present study is based on the object to formulate a protein based nanoparticles loaded with a NSAID to regulate chronic deformity which occurs due to inflammation as in case of rheumatoid arthritis, diabetic retinopathy etc.

EXPERIMENTAL

Materials and Methods:

Materials: Aspirin was purchased from Ranbaxy lab, Dewas, India, Gelatin Type B, Glutaraldehyde & n-Octanol was purchased from Himedia, Mumbai, India, Acetone, Methanol & HCl was purchased from Rankem, Mumbai, India. Carrageenan was procured from Himedia, Mumbai and Streptozotocin was procured from Sigma Aldrich, India. All supplementary chemicals were of analytical grade.

Method:The original preparation process is adopted as described by Coesteret al[12] : 1.25g of gelatin type B (Bloom225) was dissolved in 25 ml water (5% w/w) under gentle heating. A first desolvation step is commenced by adding 25 ml acetone with the drug. The supernatant containing desolvated gelatin along with gelatin in solution was rejected following subsequent sedimentation of precipitated gelatin[13]. Now, the residue was dissolved again by the addition of 25 ml water under heating and the pH was adjusted to 2.5. In situ gelatin nanoparticles were formed during a second desolvation step by drop wise addition of 50 mL acetone under stirring (500 rpm). After 10 min, 200 µl of glutaraldehyde (25%) was added to the reaction vessel to crosslink the nanoparticles. Finally, after stirring for 12 hours, the particles were refined by three-fold centrifugation (16000 g for 20 min) and redispersion in acetone/water (30/70). The purified nanoparticles were stored as dispersion in highly purified water (conductivity < 0.04 µs/cm) at 4-8 °C.

Optimization:

To achieve nanoparticles of specific size, we had to characterize the influence of various process variables in the manufacturing process in terms of particle size and poly dispersity index, keeping the ratio of crosslinking agent as constant. We expected the following factors to be most important and decided to investigate their impact on the resulting nanoparticles:

1. Temperature before the first and second desolvation step (30 – 65oC);
2. Desolvating agent addition speed (3 – 5 mL/min);
3. Amount of desolvating agent (25 – 75 ml);
4. Stirring speed (100 – 1000 rpm)

After performing the above studies; the nanoparticles were further optimized by varying the amount of crosslinking agent added and the various formulations obtained were characterized for the listed below parameters:

1. Drug entrapment
2. Drug release
3. In-vivo studies
4. Ex vivo studies
5. Stability studies

Characterization and Evaluation of Nanoparticle:

Particle size determination:

The obtained nanoparticles were suspended in distilled water by sonication and vortex mixing for 30 seconds and the particle size (Z-average mean) and zeta potential were determined by using Nano series Malvern Instruments, UK.

Surface Morphology:

Shape and surface morphology was studied using Scanning Electron Microscopy (SEM) (JSM-6500 F, Jeol, Ebersberg, Germany) at 5.0 kV and a working distance of 9.7 mm. For sample preparation gelatin nanoparticles were dispersed in acetone at a concentration of20 µg/mL and applied on a

specifically polished sample grid. The samples were vacuum-dried over 12 hours and finally metallized with a 2 nm gold layer before microscopical analysis[14, 15]. Lyophilized nanoparticles were suspended inphosphate buffer (pH 7.4) and was observedby transmission electron microscope (Philips, CM 200, Acceleration voltage 200kV, resolution 0.23 nm).

Encapsulation efficiency determination:

The entrapment efficiency of gelatin nanoparticles was determined by centrifuging the nanoparticles by using centrifuge (Remi) at 10000 rpm for 30 min. The amount of drug in the supernatant was measured by UV Spectrophotometer at 275nm. The gelatin entrapped in the nanoparticles was calculated by following formula[16];

$$\text{Entrapment efficiency} = \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of drug used in formulations}} \times 100$$

In vitro release studies:

In vitro release studies were carried out by using dialysis tubes with an artificial membrane. The prepared aspirin nanoparticles were re-dispersed in 5 ml of phosphate buffer pH 7.4 and subjected to dialysis by immersing the dialysis tube to the receptor compartment containing 150 ml of phosphate buffer pH 7.4.

The medium in the receptor was agitated continuously using a magnetic stirrer and the temperature was maintained at 37±1oC. 5ml sample from the receptor chamber was drawn at various time interval over a period of 24h and the sample withdrawn was replaced with 5ml of fresh buffer. The amount of drug released was determined spectrophotometrically at 275 nm[17].

Kinetic modeling:

Kinetics and drug release mechanism may well be better understood by applying the results of in vitro drug release into various kinetic equations like zero order (cumulative% release vs. time), first order (log% drug left behindvs. time), Higuchi's model (cumulative% drug release vs. square root of time), Peppas plot (log of cumulative% drug release vs. log time). R2 (coefficient of correlation) and k (release rate constant) values were considered for the linear curve acquired by regression exploration of the plots[18, 19, 20].

Pharmacological Study:

Animal care and handling: The experiment was carried out on Wistar albino rats of 4 months, of either sex, weighing 140-180gm. Animal models were provided by Institute of Pharmacy, Bundelkhand University, Jhansi. The animals were familiarized to the standard laboratory environments in cross ventilated animal house at temperature 25±2°C relative humidity 44 –56% and light and dark cycles of 12:12 hours, served with consistent pallet regime and water ad Libitum throughout experiment. The experiment was permitted by the Institutional Ethics Committee and as per CPCSEA guidelines (approval no. BU/Pharm/IAEC/11/035).

Chemicals:

Carrageenan was purchased from Himedia. Streptozotocin was purchased from Sigma Aldrich. All other chemicals used for this study were of analytical grade.

Carrageenan-induced rat paw edema:

Acute inflammation was caused by injecting 0.1 ml of 1 % (w/v) carrageenan in saline into the sub-plantar region of the right hind paw of each rat. The paw volume was measured plethysmometrically at 0 h, 1 h, 2 h, 3h, and 4h after the carrageenan injection. Edema was expressed as mean increase in paw volume relative to control animals.

The percentage inhibition of edema was calculated by the following equation: % inhibition of edema= 100 (1-Vt/Vc), where Vc is the edema volume in the control group and Vt is the edema volume in tested groups [21].

Experimental Design:

In the experiment, a total of 24 rats were used. The rats were divided into 4 groupscomprising of 6 animals in each group as follows:

a. Group I: Control, inject carrageenan (0.1 ml.).
b. Group II: Rats treated with Aspirin, (300 mg/kg, p.o.) before 1hr of carrageenan injection.
c. Group III: Rats treated with Formulation-1, (300 mg/kg, p.o.) before 1hr of carrageenan injection.
d. Group IV: Rats treated with Formulation-2, (300 mg/kg, p.o.) before 1hr of carrageenan injection.

Streptozotocin induced diabetes:

Diabetes was persuaded in rats by a solitary intraperitoneal injection of a newly primed Streptozotocin (STZ). 10 mg/ml STZ solution was prepared in ice-cold citrate buffer 0.1 M,

pH 4.5 and was directed at a dose of 50mg/kg-body weight. Hyperglycemia was confirmed on the third day of STZ -injection. Hyperglycemic rats with moderate diabetes (blood glucose level above140 mg/dl) were selected for the experiment. After 7 days, inflammation was induced by carrageenan in right paw and observed paw volume at 0, 1, 2, 3, 4hr[22].

Experimental Design:

In the experiment, a total of 25 rats were used. The rats were divided into 5 groups comprising of 5 animals in each group as follows:

Group I: Normal control rats received 1ml/100gm

Group II: Negative control rats received STZ, 50mg/kg, i.p. for inducing diabetes then inject carrageenan on 7th day.

Group III: Rats received STZ, 50mg /kg, i.p. for inducing diabetes and treated with aspirin (300mg/kg, p.o.) on 7th day 1hr before of carrageenan injection.

Group IV: Rats received STZ, 50mg /kg, i.p. for inducing diabetes and treated with Formulation-I (300mg/kg, p.o.) on 7th day 1hr before of carrageenan injection.

Group V: Rats received STZ, 50mg /kg, i.p. for inducing diabetes and treated with Formulation-II (300mg/kg, p.o.) on 7th day 1hr before of carrageenan injection.

Sample collection:

Blood samples were collected by tail vein and blood glucose levels were estimated using an electronic glucometer on 3rd and 7th day (Glucochek). On 7th day, the paw volume was measured plethysmometrically at 0 h, 1 h, 2 h, 3h, and 4h.

Statistical analysis:

All the values are expressed as mean ± standard error of mean (S.E.M.) and analyzed for ANOVA and PosthocTukey-Kramer Multiple Comparisons Test by employing statistical software, GraphPadInStat-3. Differences between groups were considered significant at $P < 0.05$ levels.

Ocular Irritancy Studies:

A polymer's biopharmaceutical as well as ocular tolerance properties has to be assayed before it is utilized as a drug delivery system supposed for an ophthalmic drug carrier. Hence, the formulations were tested forin vivo ocular irritancy toward the gelatin nanoparticles by means of a modified Draize test protocol[23].

Ex vivo corneal retention and permeation studies: Goat cornea was excised immediately after the animal sacrifice (local slaughterhouse, Jhansi, U.P.) and was placed in an iced (4°C) Krebs buffer which was in continuous supply of oxygen. Each cornea was placed between the donor and the acceptor chamber of diffusion cells and oxygen was bubbled into Krebs buffer in both chambers. To quantify the transport of the formulation F2 through cornea, buffer of donor chamber was replaced by 5 mL of formulation (Aspirin content 250 ug/ml). In another setup, buffer of donor chamber was replaced by plain drug solution with similar drug content to compare the result. The goat corneas were removed after 1 hr, 3hrs, 6 hrs and 12 hrs respectively from the perfusion cells from both the setup. To quantify the corneal penetration of the drug buffer from the acceptor chamber was withdrawn at half an hour interval upto 12 hrs. Corneal retention of aspirin was estimated by rinsing each tissue with normal saline, blotted dry and was relocated to pre-weighed counting vials. Vials were reweighed and the weight of the tissues was calculated. The tissues were digested. The medium was acidified by using 1 ml of 1M HCl followed by centrifugation at 15,000 rpm for 15 min.

The drug was extracted with DMSO and quantified by HPLC method. Stock solution of aspirin was produced by the addition of 10 mL of methanol to reach a final aspirin concentration of 5 mg/mL. Calibrated samples were obtained by the dilution of this stock solution in a blend of methanol/dimethylsulfoxide (DMSO): 50/50 (v/v). A second dilution was performed in Na2EDTA 0.02 M /acetonitrile: 45/55 (v/v) at pH 5.0 run isocratically. Na2EDTA 0.02 M /acetonitrile: 45/55 (v/v) was used as mobile phase with 5 pH which was flowing at a rate of 1 mL/min.

Stability studies:

The stability study was conceded out using the batch F2. Formulation F2 was divided into 3 sets of samples and stored at 4oC in refrigerator, room temperature (29oC), 45±2oC/75% RH in humidity control ovens. After 60 days, drug content of all samples was analyzed. In vitro release study of F2 was carried out after 30 days of storage[24].

Results and Discussions:

Solubility Studies: The sample was qualitatively tested for its solubility in various solvents. It was determined by taking 10 mg of drug sample in 10 ml of solvent as water, methanol, ethanol, Phosphate buffer pH 6.8, acetone and in 0.1 N HCl in small test tubes and well solubilized by shaking.

Table 1: Speed of Desolvating agent

Speed of Acetone addition (mL/min)	Particle Size (nm)	Poly Dispersity Index
0.5	273±1.98	0.058± 0.022
01	245±1.91	0.047± 0.017
02	225±1.78	0.036± 0.016
03	200±1.23	0.023± 0.016
04	214±1.45	0.027± 0.018
05	253±1.35	0.048± 0.021
06	423±2.78	0.119± 0.98

Table 2: Stirring speed

Stirring Speed (rpm)	Particle Size (nm)	Poly Dispersity Index
100	233±1.98	0.019± 0.012
200	220±1.91	0.022± 0.014
300	215±1.78	0.018± 0.015
500	200±1.23	0.017± 0.013
800	249±2.12	0.016± 0.014
1000	353±2.35	0.098± 0.038

Table 3: Entrapment Efficiency of Aspirin loaded gelatin nanoparticles

Formulation	Crosslinking Agent (GTA) (µL)	Entrapment efficiency (%)	Size (nm)	Polydispersity Index
F1	100	56.53±1.45	192±2.17	0.017± 0.012
F2	150	55.34±1.58	196±2.08	0.019± 0.013
F3	200	53.33±1.86	207±2.15	0.021± 0.016
F4	250	52.15±1.55	223±2.18	0.021± 0.019
F5	300	51.63±1.49	251±2.29	0.024± 0.014
F6	350	48.83±1.83	287±2.84	0.028± 0.018
F7	400	43.73±1.52	325±2.73	0.031± 0.017
F8	450	52.87±1.82	386±2.06	0.037± 0.019
F9	500	51.71±1.24	402±2.84	0.039± 0.020

Table 4: Correlation coefficient (R^2) of different kinetic models for Aspirin loaded Gelatin Nanoparticles

Formulations	Zero Order	First Order	Higuchi Equation	Pappas Equation
	R^2	R^2	R^2	R^2
F1	0.984	0.977	0.884	0.963
F2	0.989	0.975	0.888	0.943
F3	0.987	0.974	0.894	0.936
F4	0.987	0.982	0.898	0.961
F5	0.989	0.98	0.892	0.969
F6	0.978	0.969	0.881	0.923
F7	0.978	0.969	0.877	0.938
F8	0.984	0.972	0.873	0.954
F9	0.981	0.964	0.871	0.954

Table 5: Effect of Formulation-1 & 2 on carrageenan induced paw edema in normal rats

Groups	Paw volume (mm) (Mean±SEM)					% Inhibition
	0hr	1 hr	2 hr	3hr	4hr	
I	0.23±0.02	0.45±0.03	0.5±0.02	0.56±0.02	0.51±0.03	-
II	0.26±0.03	0.33±0.02a*	0.36±0.02a**	0.38±0.04a**	0.36±0.03a*	29.41
III	0.21±0.03	0.3±0.02a**	0.33±0.02a***	0.35±0.03a***	0.35±0.03a**	31.37
IV	0.25±0.02	0.3±0.02a**	0.33±0.02a***	0.35±0.02a***	0.33±0.02a**	35.29

All values are mean □ SEM, n = 6. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

a- Significance difference as compared to group-I (control).

b- Significance difference as compared to group-III (Reference).

Table 6: Blood glucose level on 3rd and 7th day

Groups	Treatment	Blood glucose (mg/dl)	
		3rd day	7th day
I	Control	85.33±4.381	81.5±3.512
II	Diabetic Control	161.83±4.93	170.66±4.52
III	(Aspirin 300mk/kg p.o)	159.16±6.04	171.83±1.9
IV	F1 (300mk/kg p.o.)	160.0±5.8	172.16±4.52
V	F2 (300mk/kg p.o.)	156.16±5.6	169.66±3.8

Table 7: Effect of Formulation-1 & 2 on carrageenan induced paw edema in diabetic rats.

Groups	Paw volume (mm) (Mean±SEM)					% Inhibition
	0hr	1 hr	2 hr	3hr	4hr	
I	0.18±.001	0.18±.001	0.18±.001	0.18±.001	0.18±.001	-
II	0.2±0.02	0.33±0.02a***	0.4±0.02a***	0.43±0.02a***	0.45±0.02a***	-
III	0.23±0.02	0.3±0.02	0.3±0.02a**,b*	0.33±0.02a***,b*	0.31±0.01a***,b***	31.11
IV	0.21±0.03	0.35±0.02a**	0.35±0.02a***	0.36±0.02a***	0.33±0.02a***,b**	26.66
V	0.2±0.02	0.031±0.01a*	0.31±0.01a**	0.33±0.02a***,b*	0.31±0.01a***,b***	31.11

All values are mean □ SEM, n = 6. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

a- Significance difference as compared to group-II (diabetic control).

b- Significance difference as compared to group-III (Reference).

Table 8: Entrapment efficiency of different nanoparticle formulation using Cornea

Time (h)	Amount of drug transported through cornea to buffer solution		
	Plain drug solution	F1 nanoparticle formulation	F2 nanoparticle formulation
0.5	10 ng	18 g	35.5 g
1	25ng	50 g	65 g
2	50 ng	75 g	85 g
3	100 ng	88 g	110 g
4	150 ng	125 g	125 g
6	200 ng	155 g	175 g

Table 9: Physical Stability of gelatin Nanoparticle

S. No.	Storage Condition	Time (Days)	Physical Stability (Visual Observation)		
			Color	State	Order
1	NC	Initial	NCC	NCC	NCC
2	NC	15 Days	NCC	NCC	NCC
3	NC	30 Days	NCC	NCC	NCC
4	NC	45 Days	NCC	NCC	NCC
5	NC	60 Days	NCC	NCC	NCC
6	SC	Initial	NCC	NCC	NCC
7	SC	15 Days	NCC	NCC	NCC
8	SC	30 Days	NCC	SL	NCC
9	SC	45 Days	SF	SL	NCC
10	SC	60 Days	SF	SL	NCC

NC- Normal condition (e.g. room temperature 25°C ± 2°C), SC- Stress condition (40° ±5°C, %RH- 70± 5), NCC- No change, SF- Slight fade, SL- Semiliquid

Table 10: Chemical Stability of gelatin Nanoparticle

S. No	Storage Condition	Time (Days)	Stability Test		
			Particle Size (nm)	%Entrapment Efficiency	Drug Release %
1	NC	Initial	182	56.53	70.41
2	NC	15 Days	180	55.13	69.87
3	NC	30 Days	179	54.06	69.73
4	NC	45 Days	178	52.73	68.60
5	NC	60 Days	173	51.22	68.08
6	SC	Initial	182	56.52	70.41
7	SC	15 Days	179	55.09	68.80
8	SC	30 Days	176	53.53	68.04
9	SC	45 Days	174	51.02	67.93
10	SC	60 Days	172	48.69	67.38

NC- Normal condition (e.g. room temperature 25°C ± 20°C), SC- Stress condition (40°±5°C, %RH- 70±5)

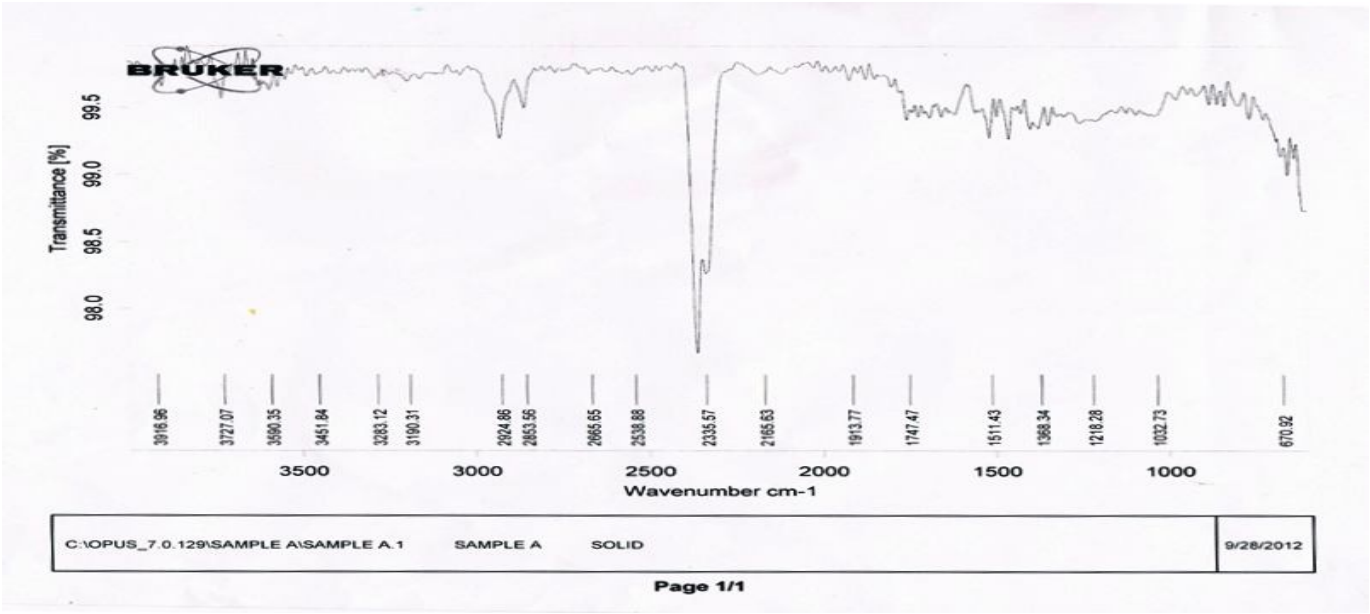

Fig. 1: FTIR Graph for pure Aspirin

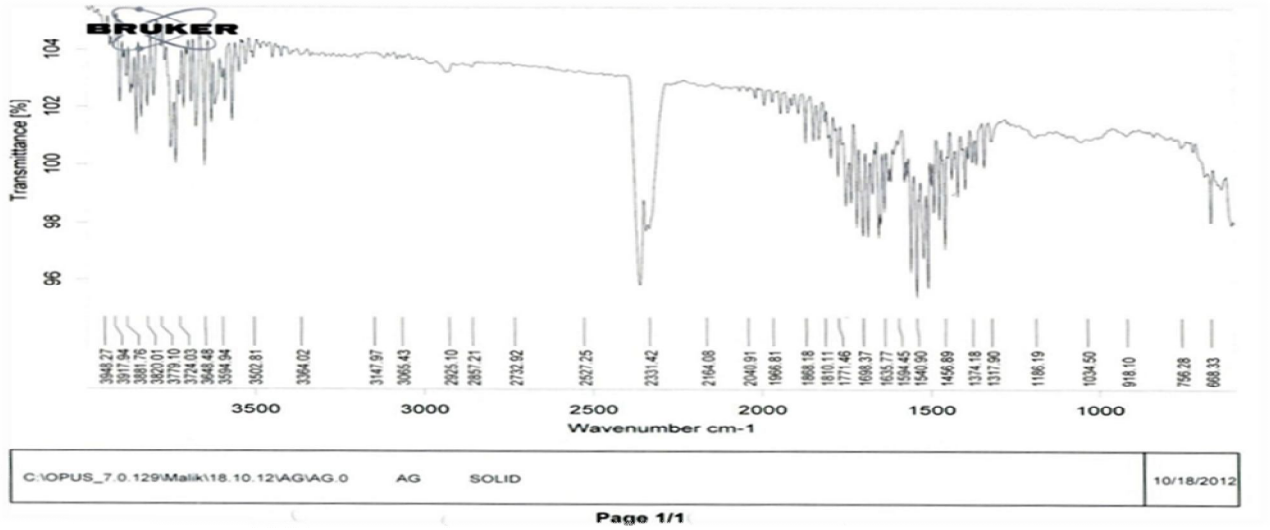

Fig. 2: Aspirin + excipient FTIR graph

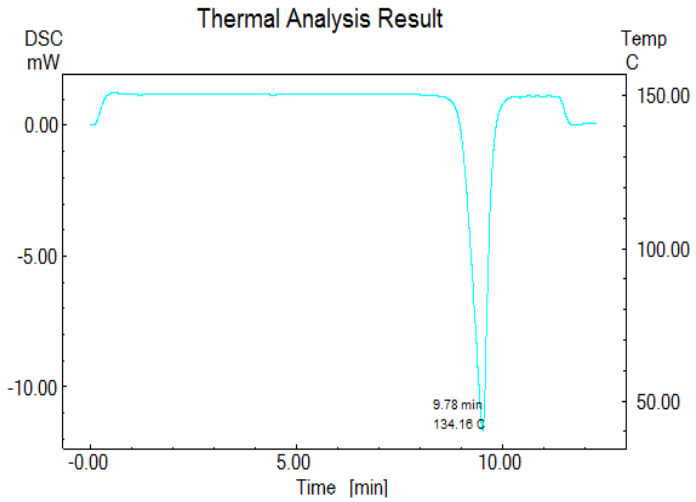

Fig. 3: DSC Curve of Aspirin

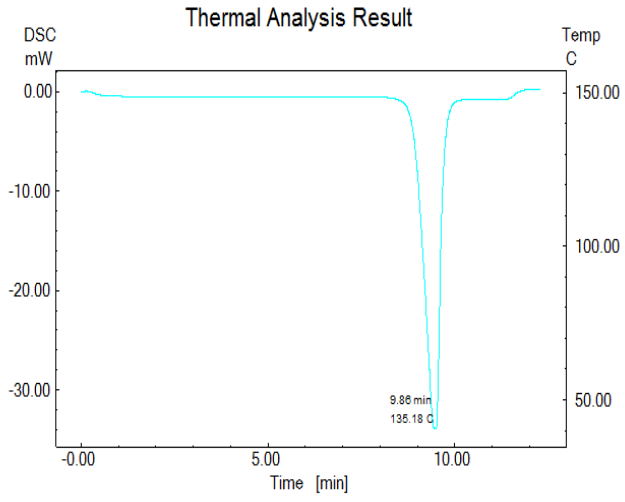

Fig. 4: Graph of Aspirin with gelatin

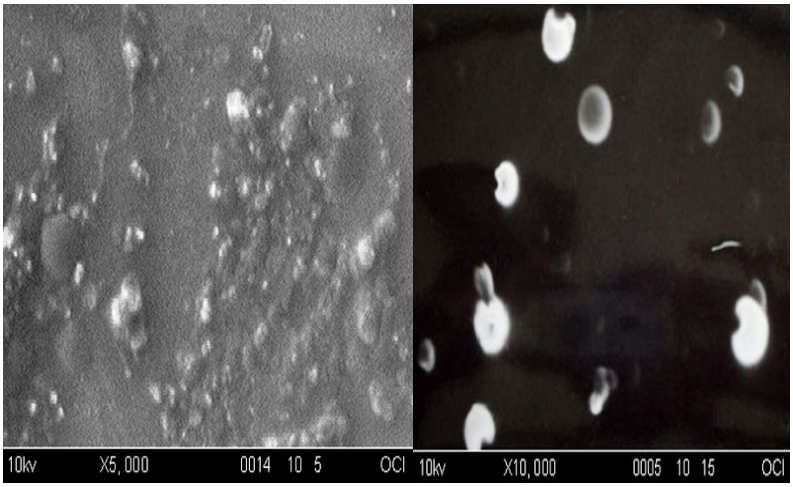

Fig. 5: SEM micrograph of gelatin nanoparticle

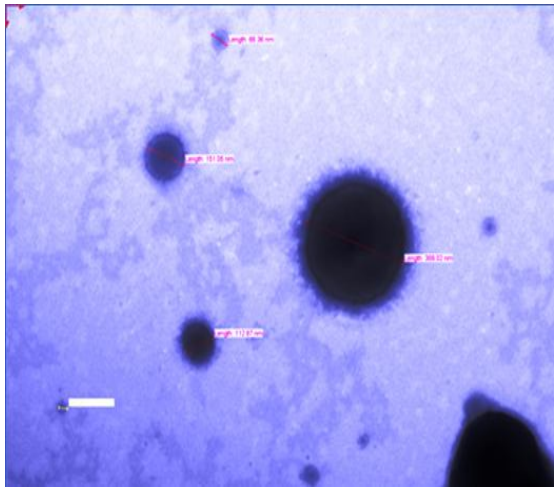

Fig. 6: TEM of Gelatin nanoparticles optimized formulation

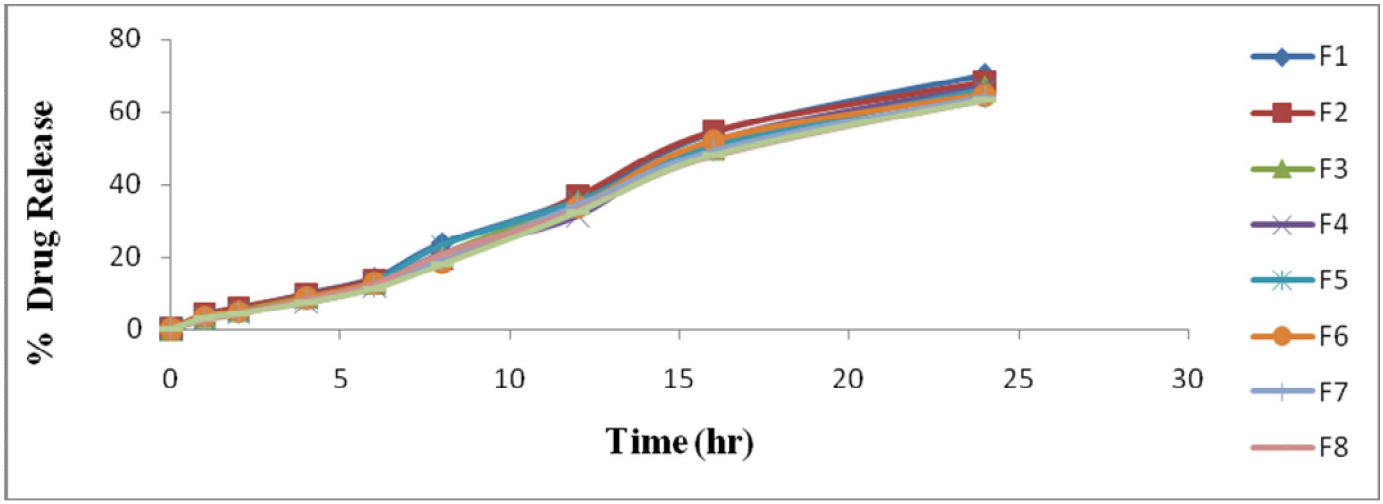

Fig. 7: Dissolution curve of Gelatin Nanoparticle

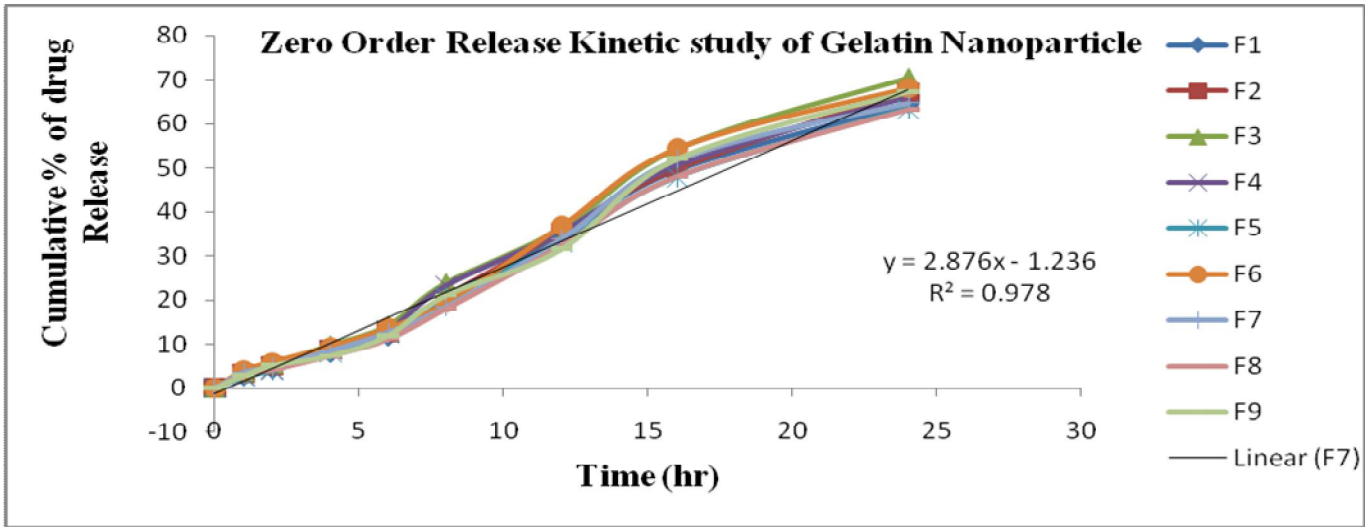

Fig. 8: Zero Order Kinetics study of aspirin loaded nanoparticle

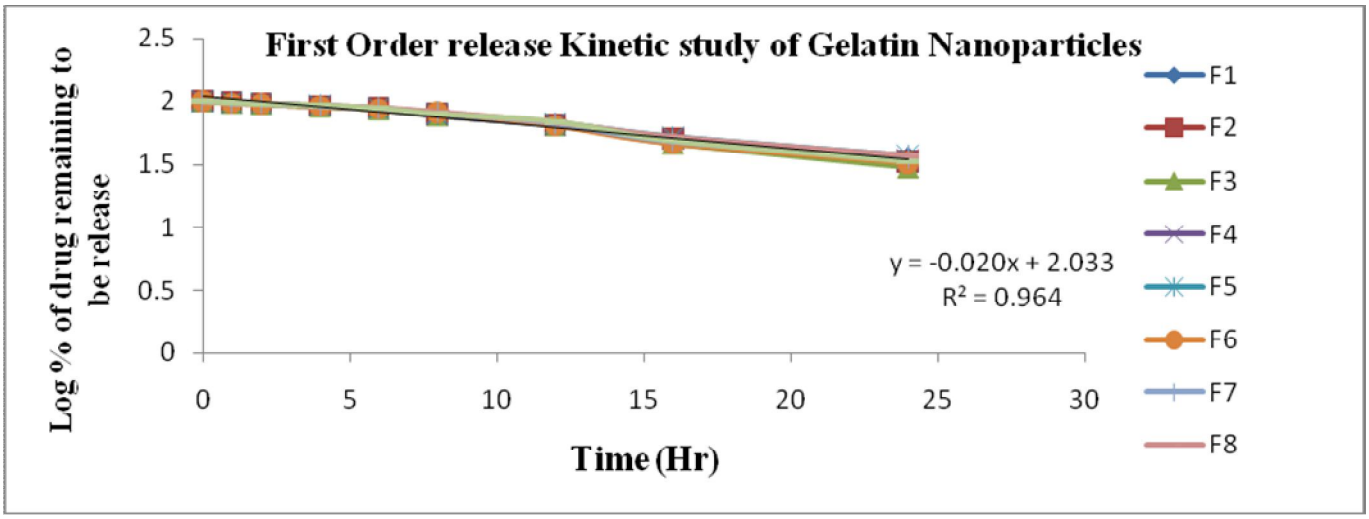

Fig. 9: First order Release Kinetics

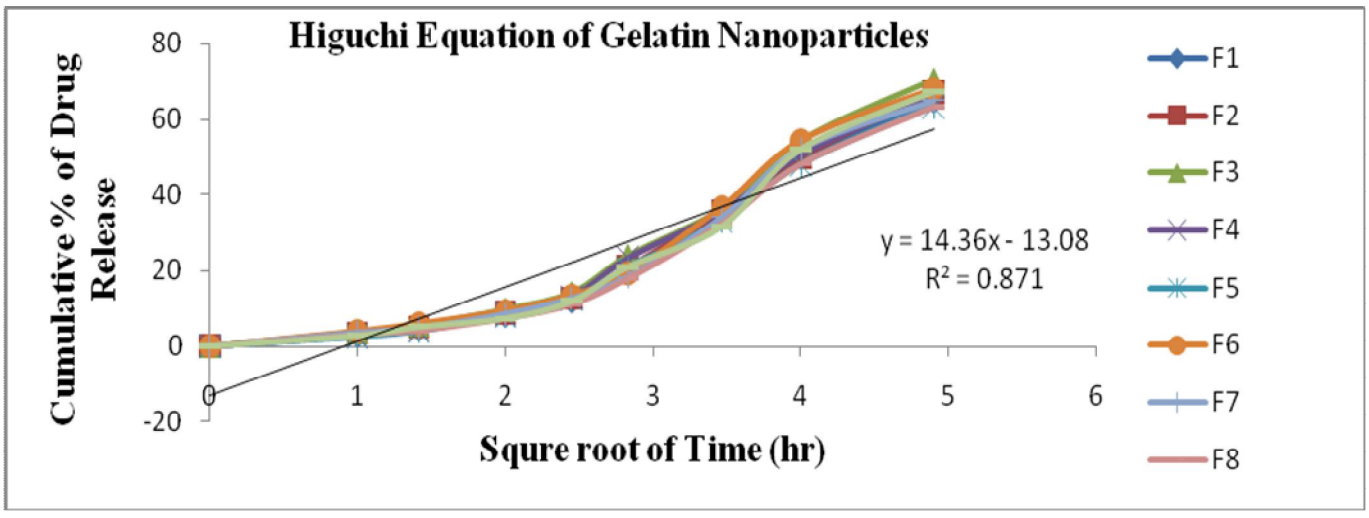

Fig. 10: Higuchi Equation

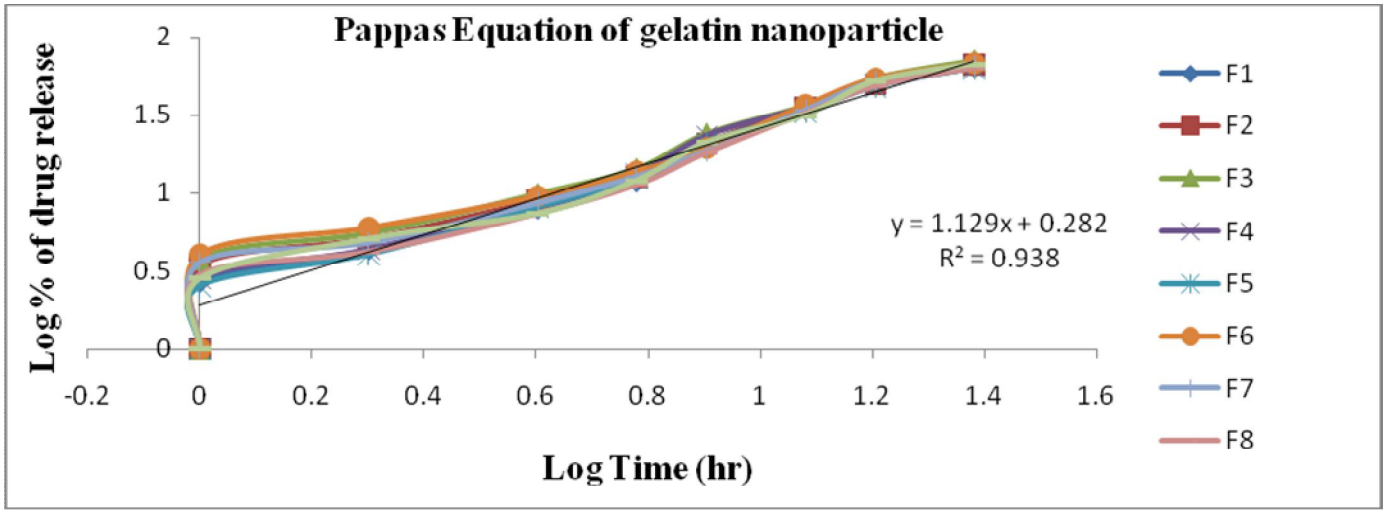

Fig. 11: Pappas Equation

Fig. 12.1: Left eye

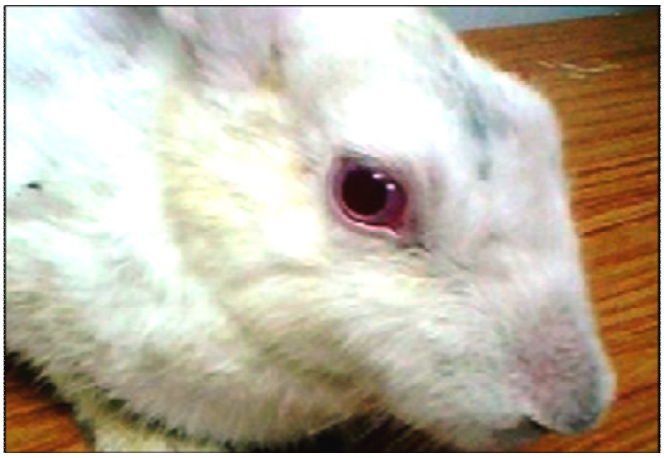

Fig. 12.2: Right eye

Fig. 12A: Effect of nanoparticle preparation in albino rabbit eye at the time of administration

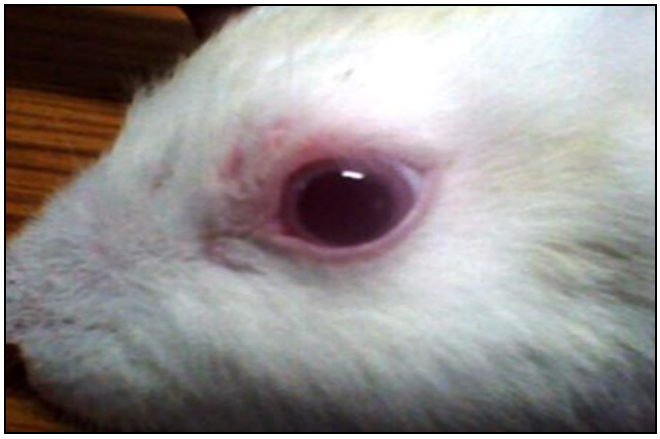

Fig. 12.1 Left eye

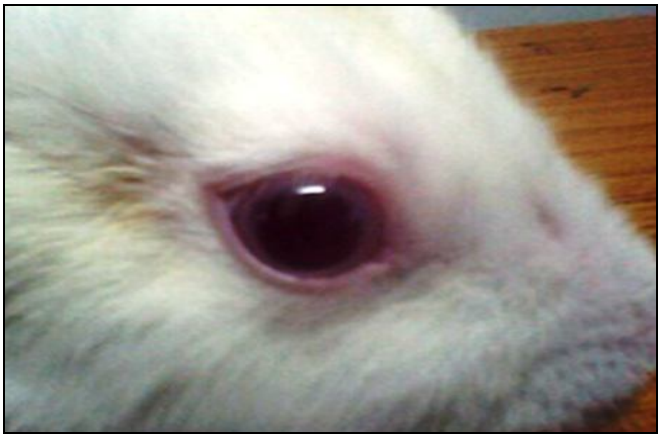

Fig. 12.2 Right eye

Fig. 12B: After administration of nanoparticle in left eye and right eye

Solubility study in different solvents at room temperature revealed that it is freely soluble in ethanol and methanol soluble in Phosphate buffer 6.8, 0.1N HCl and acetone and it is very slightly soluble in water.

Drug - polymer interaction study using FTIR: FTIR spectra of the drug matches with that of the standard and hence it can be confirmed that the drug is pure (Figure 1). Different ratios of drug and polymer were utilized to conform any interaction between them. The results showed no such interaction (Figure 2). FTIR studies were observed for pure Aspirin and aspirin with polymer nanoparticles. In FT-IR studies, the characteristic aspirin Asymmetric stretching of C-H group around 2924cm^{-1} and symmetric stretching of Aromatic C=C Bending at 1511cm^{-1} were observed in pure aspirin, suggestive of no drug-polymer interactions in loaded nanoparticles.

Differential Scanning Colorimetry (DSC): DSC thermogram of pure drug Aspirin exhibits a sharp endothermic peak at 135.18^{0}C (fig. 3). The DSC curve for physical mixture of excipients showed endothermic peak at about 134.16^{0}C (fig. 4).

Optimization: Factors like temperature and its effect before the first and second desolvation step (30 – 65^{0}C), desolvating agent addition speed (3 – 5 mL/min), amount of desolvating agent (25 – 75 ml) and stirring speed (100 – 1000 rpm) were considered during the preparation of the gelatin nanoparticles as they are supposed to influence the final formulation in terms of particle size and polydispersity index.

Temperature:

The first parameter investigated was the prevailing temperatures before the induction of the first respectively, the second desolvation step. The temperature ranges between 35^0 – 60^{0}C in the first desolvation step and the particle size range was from 273±1.98 - 253±1.35 nm with pdi ranging between 0.056±0.021 – 0.049±0.020. At the second desolvation step, the temperature ranges between 35^0 – 60^{0}C and the particle size range was from 182±1.98 - 178±1.35 nm with pdi ranging between 0.019± 0.011 – 0.019± 0.011. It is well known from the literature, that temperature has substantial effect on the specious molecular weight distribution of gelatin, due the manifestation of denaturation or renaturation processes[25, 26]. Each condition was tested in triplicate. Differing from the expectations, variations of the gelatin solutions' temperatures did not induce significant alterations in the resulting particles. At least, concerning the temperature before the first desolvation step a slight trend could be seen.

Particle size at 60^{0}C and 30^{0}C were observed to be larger in size, but particles between 50-55^{0}C were of optimal nano range and were considered for further studies. So, it can be established that the chosen standard conditions (50°C for both) also seem to represent the ideal setup as well.

Desolvating agent addition speed:

As depicted in table 1, the resulting nanoparticles were not adversely influenced up to a rate of 5 mL/min. beyond this limit, mean particle sizes along with their corresponding PIs increased exponentially, thus indicating either particle aggregation or appearance of inhomogeneous nanoparticles.

Consequently, no higher rate seems advisable if no further changes of the standard conditions (e.g. enhanced stirring) are applied. Below 5 mL/min, the process was robust contrary to variations in acetone addition rates.

Stirring speed:

As the stirring speed of the reaction is increased, the particle seems to decrease in size but as the speed is gradually increased, the particle size is increased on thermodynamic stability of the nanoparticulate formulation because of the aggregation and accumulation of the gelatin nanoparticles (table 2).

Amount of Crosslinking agent:

Crosslinker is used to prevent the in situ formed nanoparticles fromdisintegration when the desolvation agent is evaporated. For gelatin nanoparticles, glutaraldehyde (GTA) is the agent of choice, since it is a highly reactive and efficient crosslinking agent. GTA is a non-zero length crosslinker as it operates by intra-particulate bridging of residual amino groups. In the current study, gelatin nanoparticles were prepared according to the standard conditions (adjusted pH: 3.0) and varied the amount of applied GTA from 100-500µL per sample. Each condition was prepared in triplicates[27]. As depicted in table 3, the particle size increases steadily along with higher amounts of GTA applied. It can be stated in consensus with the results obtained that increasing amounts of crosslinks lead to denser

and larger nanoparticles (refer table 3). On the optimization of nanoparticles based on temperature, solvent addition speed, stirring speed; nanoparticles of optimum particle size and poly dispersity index were chosen for final optimization step with the varying amount of crosslinking agent, as the crosslinking agent is the major optimization factor in determining the size, pdi, drug release pattern and entrapment efficiency from gelatin nanoparticles.

Entrapment Efficiency:

%EE was evaluated for the various formulations (F1 – F9) by varying the crosslinking agent amount (100 – 500 µL) to achieve enhanced formulations which can be used for auxiliary assessment. The entrapment efficiency of the various formulations (F1 – F9) decreased as the amount of crosslinking agent is increased. This may be due to the effect of GTA as the cross linker amount is increased, the particle gets dense and the space for drug is minimized as GTA forms multiple hydrogen bonds with single nanoparticle. The results are summarized in table 3.

Evaluation of Gelatin Nanoparticles:

Particle Size Analysis:

SEM photomicrograph (Fig. 5) shows the presence of definite and regular nanoparticles. Bulky accretion of nanoparticle was not detected at X10000 during a microscopic examination. The mean particle size of formulation ranges from 192to 402 nm; with an average range of polydispersity index of 0.017± 0.012 to 0.039± 0.020. F1 and F2 showed better results in terms nanosized particles (see table 3) which were then utilized for a number of assessment studies. TEM images showed that particles were of unvarying size (Fig. 6). The results indicated that the particle size increased with increasing the concentration of Glutaraldehyde. Nano range particles were observed only for some explicit concentrations of glutaraldehyde (below 1.0 µg/ml).

Under the mentioned range, it appeared that the concentration of glutaraldehyde had pintsize effect on the monodispersity of the nanoparticles. Therefore, glutaraldehyde concentration increases (0.05 - 0.7 µg/ml), glutaraldehyde molecules approach each other with a limit, leading to a limited increase in intermolecular cross-linking and thus larger but still nanoscale particles are formed. Over this concentration, due to the stronger hydrogen bonding interactions leading to adequate glutaraldehyde molecules involved in the cross-linking of a single particle, nanoparticles are effortlessly shaped. Electrostatic repulsion between particles is not adequate to preserve the stability of these large particles, hence formulation F9 showed an increase in the particle size due to precipitation of the floccules.

Zeta Potential:

The mean zeta potential of nanoparticles of optimized formulations was found to be -38.5 mV, which is contemplated appropriate for stability aspects.

Drug Release:

The in vitro release of the drug by artificial biological membranes was studied. Drug release from known amounts of aspirin's loaded Gelatin nanoparticles was evaluated. The in-vitro drug release of aspirin- loaded gelatin nanoparticle was studied for 24 hours at of pH 7.4 by using a dialysis membrane.

The results have shown that 4.217% to 6.030% were released within first 2 h (at physiological pH 7.4) in the first 12hrs, F1 showed better release profile in comparison to other formulation. Data showed that F1 formulation releases 70.414 % in 24 hrs therefore selected for further studies. The release performance of drug from the aspirin gelatin matrix when studied was found that the release may have occurred through the matrix of the nanoparticles. The drug release take place by diffusion pattern, where the drug is homogeneously dispersed. All the data is shown in Fig. 7.

Release Kinetics Study of aspirin loaded gelatin nanoparticles:

There are number of kinetic models, which describe the overall release of drug from the dosage forms. Qualitative and quantitative variations in a formulation may amend drug release and in vivo performance; hence it is necessary to develop tools that expedite dosage form development by reducing the inevitability of bio-studies is each time necessary. In this regard, the practice of in vitro drug dissolution data to forecast in vivo bio-performance can be well thought-out as the rational extension of controlled release formulations[28, 29, 30].

In order to comprehend the mechanism and kinetics of drug release, the results of the in-vitro dissolution study of the nanoparticles were fitted with various kinetic equations, the following plots were made: cumulative % drug release vs.

time (zero order kinetic model); log cumulative of % drug remaining vs. time (first order kinetic model); cumulative % drug release vs. square root of time (Higuchi model) log cumulative % drug release vs. log time (Korsemeyer model) and cube root of drug % remaining in matrix vs. time (Hixson-Crowell cube root law) (see table 4, figures 8 - 11). The coefficient of determination specified that the release data was best formfitting in zero order kinetics. Higuchi equation expressed the diffusion controlled mechanism for gelatin nanoparticles. The value for the mentioned parameters are given as means ± S.D. for triplicates in each group. P≤0.05 were considered as level of significance.

Results for Anti-inflammatory studies:

Animal studies were carried out to assess the potential of the formulated nanoparticles to suppress the inflammation caused in the test animal model. The optimized formulations F1 and F2 were considered for the mentioned subject. To investigate for analgesic and anti-inflammatory activity of the test formulations, we used carrageenan induced paw edema method.

Carrageenan-induced paw edema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects. Moreover, the experimental model exhibits high degree of reproducibility. The development of edema has been described as biphasic31.The results of carrageenan induced paw edema in normal and diabetic rats are shown in tables 5 - 7. Formulation-II treated reduced the inflammation by 35.29% and Formulation-I treated reduced the inflammation by 31.37% as compared to control in non-diabetic rats.

Test formulations were effective by decreasing paw volume in diabetic rats, 31.11% in F-II & 26.66% in F-I treated group as comparison to the diabetic control group and there was 31.11% decreasing paw volume of reference treated group which is similar to the test formulations-II. Results of the study indicated that at dose 300 mg/kg body weight, both the test formulation reduced the pain and inflammation significantly.

Ocular Irritancy Studies:

The formulation was such designed to show its effectiveness on the inflamed tissues of the cornea also. The formulations were prepared in sterile conditions. To assess its effectiveness on the eye, in vivo ocular irritancy toward the gelatin nanoparticles was determined following a modified Draize test protocol. Results obtained during in vivo study exhibits that no irritational effect was observed in the animal models, hence, the formulations selected for the study can be considered beneficial (Figure 12A & B).The in vivo results F1 and F2 showed no sign of irritation or damaging effects to ocular tissues in rabbit eyes. The scores for conjunctival swelling and discharge were always zero. Iris hyperemia and corneal opacity scores were zero at all observations. The absence of in vivo irritant activity can promote the ophthalmic use of Gelatin nanoparticles.

Ex vivo Transcorneal Permeability studies:

The Nanoparticles had better retention and more persistent interaction with the ocular surface compared to plain drug solution table 8. This result indicates two possibilities: First: Gelatin nanoparticle releases the drug in a sustained releasemanner. Second: It is evident that intactcorneal tissues are less permeable than inflammatory tissue. If the drug is crossing the intact tissue, it will surely cross the inflammatory tissues with a greater concentration. The analogous results were reported earlier by Goldblum et al., 2002[32].

The prolonged ocular retention of the Nanoparticle compared to solution is in good agreement with a previous work that showed prolonged corneal retention of colloidalparticles[33]. The results also specified that the concentration of Aspirin in cornea remained fairly constant for up to 6 h. Plain drug solution had less retention (25 ng at 1 hr.) and drug level was decreasing with increasing time (4.8ng at 12 hrs). While almost 50%and 85% of drug was penetrating at 12 hrs of nanoparticle of F1 and F2 formulations respectively. Penetration of drugwas more in case of F2 nanoparticle maybe due to greater pervasion and zeta potential of these nanoparticles.

Stability Studies:

Stability of Gelatin Nanoparticle was performed in Environmental test chamber (Company Dolphin). F1 showed the best release in in vitro release study. The physical and chemical stability studies were carried using the optimized formulation F1. The constancy of aspirin loaded nanoparticles was evaluated in terms of its particle size, entrapment efficiency and drug release shown in tables 9

and 10. Nanoparticles formulations were stored at room temperature (25°C) and at 40°C for the period of 60 days[34]. It remained stable during the specified duration of time.

CONCLUSION:

The current work embroils formulation and evaluation of aspirin loaded gelatin nanoparticles by two-step desolvation. FTIR and DSC studies showed that the drug was stable in nature during formulation.

Effect of various experimental variables like temperature, stirring speed and concentration of cross-linking agent (glutaraldehyde) were perceived. It was observed that with the increase of stirring speed, the stirrer caused high turbulence, which resulted in foaming and adhesion of polymer to the wall of the container. On increasing the GTA concentration, drug entrapment decreased. The entrapment efficiency of nanoparticles containing varied concentration of cross linker (glutaraldehyde) shows entrapment of drug in range of 51.74 -56.53 % and the particle size was found in range of 192 nm to 402 nm as the concentration of GTA was increased, entrapment efficiency decreased thereby showing increase in particle size of the nanoparticles. Thus, the formulation F1 registered maximum entrapment of 56.53% and particle size 192 nm.

In vitro release profile was applied on several kinetic models in order of zero order, first order, Higuchi equation and Peppas equation. Anti-inflammatory scrutiny of the research formulation showed agreeable results in terms of suppression of the paw edema in the selected animal model. Ocular irritancy test inveterate that the formulation is non-irritant to the animal eye and can be utilized for ophthalmic purposes. Ex vivo study on goat eyes exposed satisfactory result of drug penetration and retention at corneal tissues. The stability study was performed in stability chamber for 2-month period. It shows that the formulation was stable for all parameter after 60 days.

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