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Formulation and *In Vitro- In Vivo* Evaluation of Sustained Release Chitosan Microspheres containing Mefanamic Acid

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Abstract:

The aim of the present work was to prepare and characterise mefenamic acid (MA) microspheres using chitosan as polymer prepared by emulsion cross linking method by using gulteraldehyde as cross linking agent. MA is a potent non-steroidal anti-inflammatory drug (NSAID), has low oral bio availability due to poor aqueous solubility and insufficient dissolution, so it is used in high dose which leads to adverse drug reactions (ADRs) associated with NSAIDs such as gastrointestinal (GI) effects and renal effects of the agents. The prepared formulations were characterized for their entrapment efficiency, particle size, surface morphology and release behaviour. The mean particle size of microspheres ranged from 75 to 225 μm and encapsulation efficiency ranged from 72.72 to 95.88% (w/w). Scanning electron microscopy of microspheres revealed a spherical and uniform appearance with rough surface. Mechanism of release was found to be Higuchi type. *In vivo* study of microspheres in albino wistar rats demonstrated significant analgesic and anti-inflammatory activities of microspheres for longer period of time compared to the parent drug. This study indicated that chitosan microspheres containing MA could be prepared successfully by using an emulsion cross linking method, which could provide sustain the release of drug and also reduce the side effects of this drug.

Keywords: Chitosan, Mefanamic Acid , Analgesic Activity.

1. INTRODUCTION:

Most of the orally administered anti inflammatory drugs are effective in treating various pain conditions and also they are associated with many adverse effects like gastrointestinal bleeding when administered in high doses. The inhibition of the biosynthesis of prostaglandins by blocking the activity (selectively and/or non-selectively) of two cyclooxygenase enzyme (COX-1 and COX-2) is the principal mechanism of action of all NSAIDs (1). Mefenamic acid (MA) is primarily used as analgesic in muscle, joint and soft tissue pain where strong inflammatory action is not needed. It may be useful in some cases of rheumatoid and osteoarthritis but has no distinct advantage (2). It could be used as a therapeutic agent in Alzheimer's disease since it improves learning and memory impairment in an amyloid β peptide (Aβ1–42)-infused Alzheimer's disease rat model .As it is having potential to cause gastrointestinal bleeding when administered in high doses it is formulated in microspheres to achieve the controlled release there by reducing severity of the gastrointestinal problems (3).

In general the multiparticulate systems perform better in vivo than single unit systems, as they spread out through out the length of the intestine causing less irritation, and give a more reproducible drug release .The basic idea of developing multiparticulate drug delivery system is to provide maximum drug concentration .(4-6) Chitosan is biodegradable natural polymer with great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesion. The suitability and performance of chitosan as a component of pharmaceutical formulations for drug delivery applications has been investigated in numerous studies. These include controlled drug delivery applications, use as a component of mucoadhesive dosage forms, rapid release dosage forms, improved peptide delivery, colonic drug delivery systems, and use for gene delivery. (7-9)

The aim of present investigation is to formulate MA chitosan microspheres (CSMS) and to compare the potency of pure drug with the controlled release formulation.

2.MATERIALS AND METHODS:

Chitosan was procured from Central fisheries research institute cochin, MA was generous gift sample from Alkem laboratories Mumbai, Gluteraldehyde, liquid paraffin ,chloroform were purchased from Merck India ltd,

2.1 Method of Preparation of microspheres:

A solution of chitosan was prepared in aqueous acetic acid solution and varied amounts of amount of drug in 1:2 &1:1 drug polymer ratio was suspended in it. The suspension was emulsified in liquid paraffin and sunflower oil containing 2% w/v span 85. The membrane was formed by interfacial

Table No.1 The formulation variables used in preparation of microspheres

Contents	*F1*	*F2*	*F3*	*F4*	*F5*	*F6*
Drug (Mefanamic acid)	500mg	1000mg	500mg	1000mg	1000mg	500mg
Chitosan	1000mg	1000mg	1000mg	1000mg	1000mg	1000mg
Liquid paraffin	100ml	-	100ml	100ml	100ml	100ml
Sunflower oil	-	100ml	-	-	-	-
Gluteraldehyde Chloroform saturated solution	6ml	6ml	6ml	6ml		
Gluteraldehyde Toluene saturated solution	-	-	-	-	6ml	6ml
Span-85	2% w/v	2% w/v	2% w/v	2% w/v	2% w/v	2% w/v

polymerization of chitosan following addition of gluteraldehyde-saturated chloroform and gluteraldehyde –saturated toluene (6ml). The emulsion was formed in 250ml beaker, under constant stirring by means of simple paddle at 1750 rpm at room temperature. Solidified microspheres were separated from the reaction mixture by vacuum filtration, washed several times with chloroform and methanol and dried for 24 hours at room temperature and stored in a desiccator. The various formulation parameters are given in (Table No-1).

2.2 Scanning electron microscopic analysis

The shape and surface characteristics of microspheres were analyzed by scanning electron microscopy (SEM). Sample was dusted on a double-sided adhesive tape applied previously to an aluminium stub. Excess sample was removed and stub coated (Polaron Sputter 7040) with 30 nm layer of gold-palladium observed with a scanning electron microscope (Leo 0430, Leica Cambridge Ltd., UK).

2.3 Fourier transforms infrared microscopy:

Fourier transform infrared (FT-IR) spectra of the samples were obtained in the range of 400 to 4,000 cm−1. IR spectral analysis of pure mefanamic acid, chitosan, formulation were carried out.

2.3 Micromeritic properties

The average particle size of the microspheres was determined by using optical microscopy. The flow properties and packing properties were investigated by measuring the angle of repose, tapped density and bulk density.

2.4 Drug entrapment:

Accurately weighed microspheres equivalent to 100mg of drug was dissolved in 25ml of 75% methanol and sonicated for 3 min. The solution was then filtered, diluted suitably and analyzed for drug content spectrophotometrically at 257 nm. The percentage drug entrapment was calculated as:

% Drug Entrapment = Practical drug loading/ Theoretical drug loading X 100 (1)

2.5 Invitro Dissolution Studies

Dissolution test was performed in USP XXIII dissolution test apparatus by paddle method. 250 mg of MA equivalent microspheres were placed in the dissolution apparatus and were evaluated for in vitro dissolution studies. The pH of dissolution media were kept 1.2 for 2 hr using 0.1N HCl, then 480ml of phosphate buffer and 20ml of 2MNaOH added to adjust the pH to 7.2 and maintained up to 18 hr. Aliquots samples were withdrawn at specified time intervals and replaced with same volume of fresh media, filtered and analyzed spectrophotometrically (Shimadzu 1600) at 277 nm for cumulative drug release. 5ml of each sample were withdrawn from the dissolution medium at various time intervals and replaced by an equal volume of dissolution medium. After filtration and suitable dilution, the samples were analyzed spectrophotometrically. Concentration of MA in sample was calculated based on calibration curves of MA in both the acidic 0.1NHCl media (n=3, R^2 = 0.999), and basic media (n=3, R^2 = 0.998).

2.6 Stability Studies:

The stability protocol was designed based on the ICH guidelines. The microspheres

formulations chosen were stored at 30 ± 20 C and 65 ± 5% RH for a period of 12 months and at 40 ± 20 C and 75 ± 5% RH for a period of 6 months. The stored samples were tested for their drug content and for any physical change. The testing was carried out at 0, 2, & 4 months for accelerated storage condition and at 3-month intervals for a period of 6 months for long-term storage condition.

2.7 In vivo evaluation

Procurement, identification and housing of animal Albino rats of wistar strain (200-250 g) of either sex were procured from the central animal house of the institute. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 2°C; relative humidity 60-70%) in a 12 hr light dark cycle. The rats were given a standard laboratory diet and water ad libitum. Food was withdrawn 12 hr before and during the experiment. Protocols were approved by the Institutional Animal Ethics Committee, registered under CPCSEA.

2.7.*1 In vivo* Analgesic activity

Tail immersion test:

The hot water tail immersion test unit serves to assess the tail flick reaction of mice and rats when their tail is immersed in a constant temperature bath. The animal's tail is then immersed into the water bath. The temperature range is from room temperature to 55 degrees Celsius. When the animal reacts by flicking their tail you can stop the timer by either the front panel pushbuttons or footswitch that is supplied, this unit will store and display the time of reaction. The animals were divided into three groups MA (100 mg/kg) solution in 0.1% tween 80 was used as standard drug. The results are expressed as the mean ± SEM. and statistically analyzed using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be significant. (11-12).

***2.7.2 In vivo* anti- inflammatory activity studies**:

The animals were divided into three groups. Acute inflammation was produced by subplantar injection of 0.1 ml of 1% suspension of carrageenan in normal saline, in the right hind paw of the rats. After induction of inflammation, drug was administered after one hour of oral administration of the drug, the paw volume was measured plethismographically at a time interval of 0, 0.5, 1, 2, 4, 6, 8, 10 hr. The difference between the two readings was taken after carrageenan injection as the volume of oedema and percentage anti-inflammatory activity was calculated. MA (100 mg/kg) solution in water was used as standard drug. (12-14)

2.7.2.3 Ulcerogenicity Index:

Gastrointestinal toxicity of the prepared formulation was measured and compared with the parent drug by measuring ulcer index [18]. The formulation was suspended in 10 ml of 2% w/v suspension of acacia. Measured volume of the suspension containing MA was administered orally to the test group daily for 5 days. The albino rats (100-200 g) were fasted after the administration of last dose, thereafter they were sacrificed by decapitation and the stomach was removed, opened and washed with distilled water. The lesions on the gastric mucosa were counted by visual examination using a binocular magnifier. Ulcers greater than 0.5 mm were recorded. The ulcer index (UI) was calculated by severity of gastric mucosal lesions which are graded as grade1 - less than1mm erosions, grade 2 - 1-2 mm erosions and grade 3 - more than 2 mm erosions. The UI was calculated as

UI = [1× (number of lesions of grade 1) + 2× (number of lesions of grade 2) + 3× (number of lesions of grade 3)]/10

3. RESULTS AND DISCUSSION:

MA CSMS were prepared by using gluteraldehyde as cross linking agent. .Six different formulations using different drug-polymer ratios and different external phases were prepared. Gluteraldehyde cross-linked micro spheres were pale yellowish in colour. The average particle size was in the range of 141.4μm - 208.6μm (table.2). The particle size of micro spheres prepared by using sun flower oil was found to be larger than compared to micro spheres prepared by using liquid paraffin. Hausner's ratio of the prepared micro spheres was found to be in range of 1.231-1.490 which indicates free flowing properties off micro spheres (Table No 2).

Table no.2 Evaluation of Chitosan microspheres

Batches	Mean Diameter(µm)	HAUSNER RATIO (Td/Bd)	% Drug loading	Entrapment Efficiency (wt %)
F1	141.4 ± 3.19	1.273	22.79 ± 2.22	92.4 ± 1.23*
F2	208.6 ± 3.17	1.389	16.29 ± 1.55	97.8 ± 1.68
F3	163.8 ± 2.78	1.126	18.55 ± 6.25	87.9 ± 1.56
F4	152.6 ± 2.89	1.231	21.85 ± 6.12*	85.6 ± 1.78
F5	180.6 ± 3.02	1.224	22.48 ± 2.66	94.2 ± 1.59
F6	172.2 ± 2.54	1.257	19.25 ± 5.18	88.8 ± 1.34

Table No .3 Kinetic analyses of release data

Formulatio ncode	*Zero order*		*First order*		*Higuchi matrix*		*Peppas koresmeyer*		*Hixon Crowell*	
	r	*K1*	*r*	*K2*	*r*	*K3*	*R*	*K4*	*r*	*K5*
F1	0.9596	5.7125	0.9034	-0.068	0.9952	25.9033	0.9545	0.5110	0.9498	-0.170
F2	0.9810	5.2153	0.9504	0.0605	0.9960	23.9017	0.9816	0.4728	0.9977	-0.166
F3	0.9704	5.55	0.9621	0.0594	0.9942	25.3183	0.9664	0.5306	0.9953	0.1544
F4	0.9696	5.3786	0.9704	0.0508	0.9966	24.5011	0.9655	0.5631	0.9962	-0.1383
F5	0.9900	5.4346	0.9668	0.0674	0.9952	25.0324	0.9814	0.4771	0.9954	-0.166
F6	0.9828	5.3312	0.9333	-0.069	0.9960	26.1459	0.9795	0.5506	0.9941	-0.165

Infrared spectra of mefenamic acid . MA CSMS showed characteristic peaks at 1255 cm-1 (-OH group bending and vibrations of COOH), 1647 cm-1 (N-H stretching vibration), 1572 cm-1 (C=O stretching), 1504 cm-1(Aromatic C-H plane deformation), 1163 cm-1 (Aromatic-O-CH3) and 757(Aromatic C-C vibration for ortho substitution). All the drug loaded chitosan microspheres showed similar peaks that concludes no interaction between drug and additives. (Fig No.1)

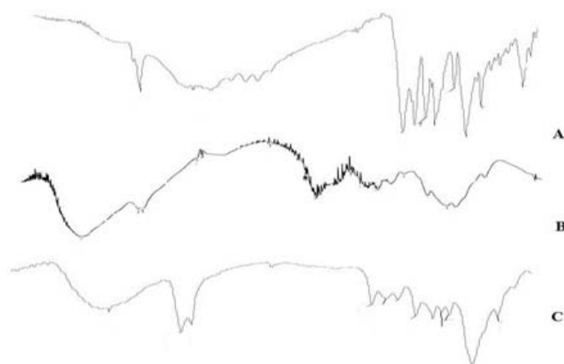

Fig No 1: A (Mefanamic acid), B (Chitosan), c (Microspheres formulation).

The drug entrapment efficiency analysis showed that entrapment of drug for different formulations of micro spheres ranges from 85.6 - 97.8% (Table No.2) which indicates high drug entrapment in the prepared formulations. Scanning electron micrograph is shown in Fig.No.2. The surface of the loaded microspheres appeared spherical but rough.

The release profile of MA from CSMS is shown in (Figure No.2.) All the microspheres showed biphasic release, initially a fast release followed by a slower release. Release profile followed Higuchi model (table No-3).

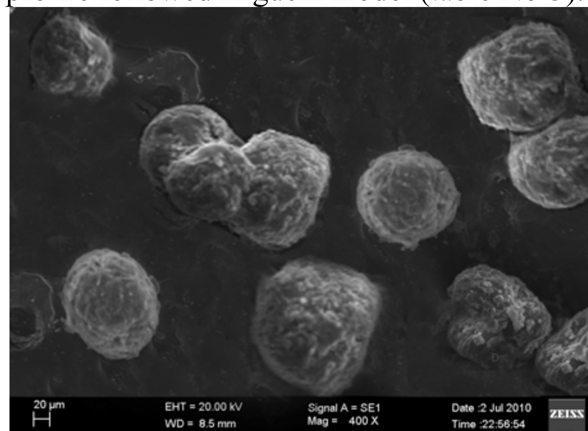

Fig No 2: The SEM analysis photographs of Chitosan Microspheres

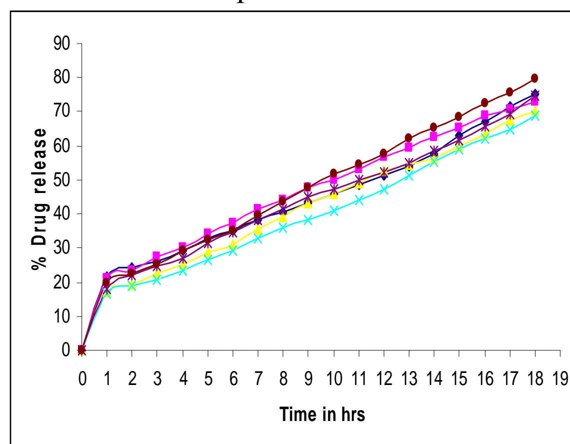

Fig No 3. In vitro dissolution profile of microspheres in 0.1N HCL

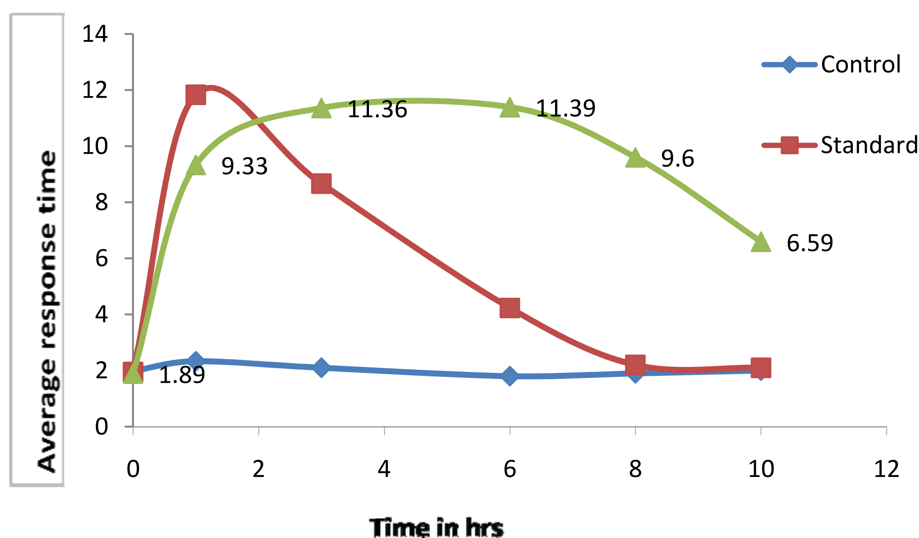

Fig No 4 : Plot of Average Response time vs post drug administration time of Optimized formulation.

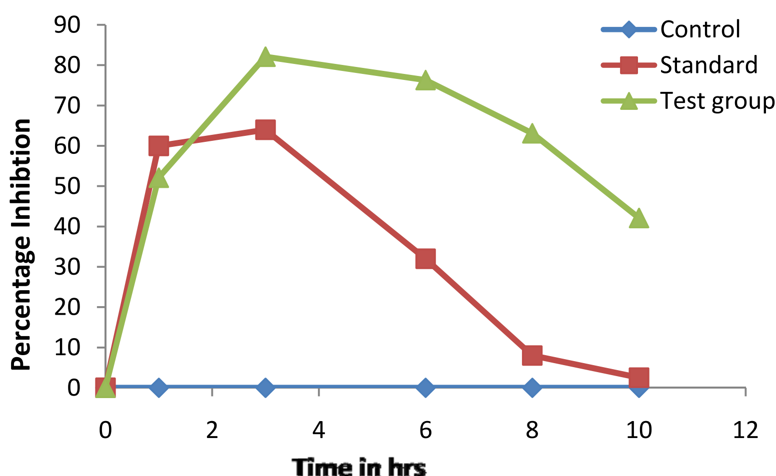

Fig No 5: Plot of Percentage inhibition in paw volume against post drug administration time of optimized formulation .

At higher drug loading drug release was fast because of entrapment of crystals in the matrix may lead to the crystals being more hydrophilic leading to enhanced wetting by the solvent It appears that mechanism of drug release from microspheres was diffusion controlled. The formulation showed minimal changes in particle size only under long term stability study with no appreciable change in drug content proving good stability of the product conducted both in accelerated and long term stability studies.

3.1 *In vivo* Analgesic Activity

Analgesic effect against thermal noxious stimuli may be elicited through opoid receptors or through modulation of several neurotransmitters involved in relevant phenomena. After administration of pure drug a rapid increase in the reaction time period was observed within 1 hr of oral administration With pure MA analgesic action was only observed up to a period of 6 hrs after action hr. In case of MA microspheres reduction in reaction time was initially slow and it started to increase significantly after 1 hr, and reached to maximum reduction of within 6 hr, after oral administration. Significant % inhibition after oral administration of MA, was maintained only from 1 - 6 hr where as in case of microspheres, it was maintained for a period of 2 - 10 hr ($p< 0.001$). The oral doses of 100mg /kg elicited a significant analgesic activity as evidenced by increase in latency time on comparison with negative control at the end of 15, 30, 60, 120 min. The increase in latency time was found in a dose dependent manner and was found to be very significant at the dose of 100 mg/kg. (Fig No.4)

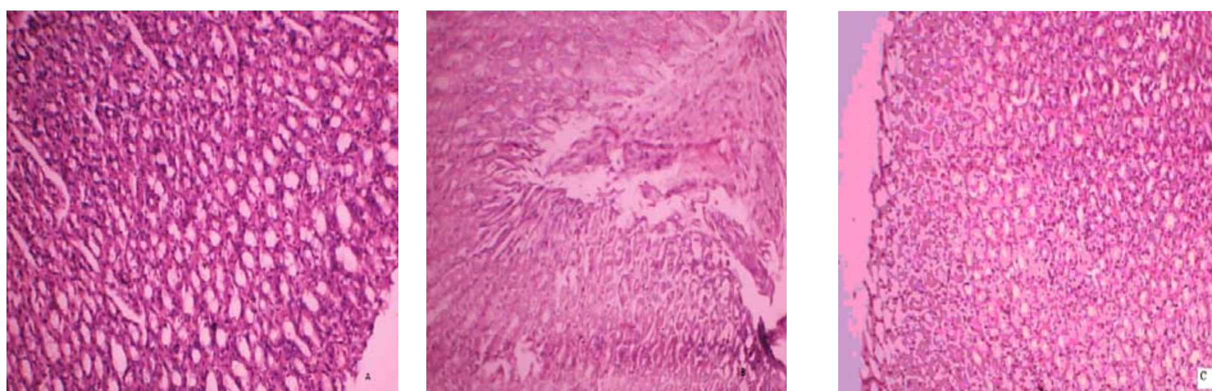

Fig No 6 .A) Healthy control B) Ulcer control showing mucosal injury characterized by MA and massive mucosal infiltration of inflammatory cell C) Treated with Chitosan Microspheres.

3.2 Carrageenan induced paw oedema method

Carrageenan-induced hind paw oedema is the standard experimental model of acute inflammation. The present study demonstrated that, the MA microspheres induced time dependent reduction of paw edema in rat, and produced significant inhibition of paw oedema for a longer period, as compared to standard drug, in dose of 100 mg/kg by oral route. Treated animals showed significant increase in percent inhibition of paw volume, compared to the control group ($p < 0.05$). Overall, the anti-inflammatory activity of pure drug was maintained only for 0.5 hr, which was fond to be subsided by 6 hr. But in case of our mcrospheres the was 42.1% at the end of 10 hr, which was maintained almost up to 12 hr and thus was significant.(Fig No.5)

Ulcerogenicity Index : The ulcer index for MA pure drug was observed to be in a higher range when compared with the ulcer index of MA microspheres . (Table No.4) A normal histological finding was observed for the samples of the control group rats. Small hemorrhagic areas and patches of inflammatory cell infiltrations were present in the lumen of the glands and lamina propria when treated with parent drug, but normal histological findings were displayed with chitosan microspheres. (Fig No.6)

Table No: 4 : Mean ulcerindex in rats

Animal group	*Pretreatment*	*Ulcer index*
1	Control	-
2	Standard	20.4
3	Formulation	12.2

4. CONCLUSION

Microspheres were prepared successfully using solvent evaporation method. The surface structure of the microspheres was spherical and rough. Optimization of all the parameters was required to obtain a better formulation with good encapsulation efficiency and reduced size. Release rate of Mefanamic acid from the microspheres were dependent on combination of polymer and the amount of polymer used. Release pattern was found to be of Higuchi type. The in vivo study demonstrated significant analgesic and anti inflammatory activity of as well as reduction in ulcer index of the when compared to the parent drug.. Sustained release without initial peak level achieved with these microsphere formulations can reduce the dosing frequency, decreased side effects and improve patient compliance.

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