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FORMULATION AND EVALUATION OF SERRATIOPEPTIDASE MICROSPHERES USING EUDRAGIT RS100 POLYMER

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ABSTRACT

Inflammation is nature's double-edged sword, characterized by pain and swelling, is triggered as a healing response in the body when it is injured or attacked by negative bacteria and viruses. Once the body recovers, the inflammation goes away. However, for tens of millions of worldwide, with disorders such as rheumatoid arthritis, sinusitis, bronchitis, fibrocystic breast disease, and carpal tunnel syndrome, the inflammation does not go away. The conventional treatment for inflammation disorders also has dark side serious side effects. Fortunately, there is a natural medicine serratiopeptidase used as an effective alternative to steroids and NSAIDs without serious side effects. Serratiopeptidase is acid labile drug thereby decreasing stability in gastrointestinal tract. It is protein in nature and claimed to be effective orally but due to its large molecular size show reduction in bioavailability. Therefore to reduce frequency of dosing as well as to increase bioavailability and enable better compliance, formulating sustained release dosage form is necessary. Serratiopeptidase microspheres have been formulated by emulsion solvent evaporation technique using polymer Eudragit RS100 and evaluated for possible anti-inflammatory potential.

Key words: Eudragit RS100, Serratiopeptidase, bioavailability, microspheres, anti-inflammatory activity.

INTRODUCTION

Inflammation is the double edged sword of nature characterized by pain and swelling,

is a protective reaction of the vascularised tissue to injury, intended to eliminate the cause of cell injury as well as necrotic cells and tissues resulting from the injury and to initiate the repair of damage done to the tissues (1-3). There are various components to an inflammatory reaction that can contribute to the associated symptoms and tissue injury. Edema formation, leukocyte infiltration and granuloma formation represent such components of inflammation (4,5). Edema formation in the paw is the result of a synergism between various inflammatory mediators that increase vascular permeability and the mediators that increase blood flow (6). Acute and chronic inflammatory disorders like rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, osteoporosis, migraine and gout are one of the most important health problems in the world. Although numerous agents like steroids as well as non steroidal anti-inflammatory drugs (NSAIDs) are known to treat inflammatory disorders, their prolonged use often leads to metabolic toxicity, ocular toxicity, immune suppression, bone marrow depression, gastric hemorrhage, peptic ulceration, include weight gain due to disrupted metabolism, increased blood sugar, loss of bone, osteoporosis, joint damage, cataracts, thinning of skin, slow wound healing, high blood pressure, emotional disorders, fluid retention, and suppression of normal adrenal function. (7).

Fortunately, there is a natural alternative to steroids and NSAIDs that is effective without serious side effects. Serratiopeptidase or serrapeptase is a protein (proteolytic) enzyme isolated from the non-pathogenic *enterobacteria Serratia* E15 found in intestine of silkworms. It has been used successfully for almost 40 years in Japan and Europe for pain and inflammation due to arthritis, trauma, surgery, sinusitis, bronchitis, carpal tunnel and painful swelling of the breasts. There is some preliminary indication that it may be useful for atherosclerosis (8). Serratiopeptidase acts in three ways to reduce inflammation; it breaks down insoluble protein byproducts of blood coagulation and thins the fluids formed from inflammation and injury as well as facilitating the rate of tissue repair process. Serratiopeptidase eases pain through blocking the release of pain inducing amines (9-11). This enzyme is absorbed through the intestines and transported directly into the bloodstream (12). However, if the enzyme is consumed in unprotected capsules or tablets it is destroyed by the acid in the stomach before it gets to the small intestine. Serratiopeptidase is reported to undergo acid hydrolysis at gastric PH thereby decreasing stability in gastrointestinal tract. Apart from the stability problems, peptides are not well absorbed from the lumen of the gastrointestinal tract due to their large molecular size and have high sensitivity to brush border peptidase activity. It is revealed from the literature survey that Serratiopeptidase has

biological half-life of 2 hours. Its bioavailability is 70-72%. Usual initial dose of Serratiopeptidase is 10 mg three times a day. Therefore to reduce frequency of dosing as well as to increase bioavailability and enable better compliance, formulating sustained release dosage form is necessary. Microencapsulation is one of the widely used techniques for sustaining drug release. This multiparticulate reservoir system is more reliable in their biopharmaceutical behavior. Upon ingestion, these particles mix with chyme, pass the pylorus at the stomach exit unhindered and spread over a large section of the intestinal tract. In order to obtain an ideal therapeutic outcome, serratiopeptidase must be protected and delivered to the intestine intact. Therefore present study was the endeavor to develop, formulate serratiopeptidase microspheres by emulsion solvent evaporation technique using polymer Eudragit RS100 and evaluated for their physical, chemical parameters and anti-inflammatory activity.

MATERIAL AND METHOD

Material

Eudragit RS 100 was provided by Evonik Degussa India Pvt Ltd, Mumbai. Serratiopeptidase was gifted by Advance Enzyme Technologies Ltd, Nashik, India. All other reagents were analytical grade and used as such.

Animals

Adult male Albino Wistar rats (150-200 g) were used to study the anti-inflammatory activity. The animals (six per cage) were maintained under standard laboratory conditions (light period of 12 hrs/day and temperature 27°C±4°C), with access to food and water ad libitum. The experimental procedures were carried out in strict compliance with the Institutional Animal Ethics Committee approval no. IAEC/May 2012/03. All experiments were performed in the morning according to the guidelines for the care of laboratory animals (13).

Preparation of Serratiopeptidase (STP) loaded Eudragit RS100 Microspheres

Microspheres were prepared by a method based on the emulsion solvent diffusion technique reported by Kawashima et al with a few modifications. Methanol and dicloromethane were used as solvents for STP and Eudragit RS100 respectively. Acetone water-miscible lipophilic solvent was used to reduce interfacial tension at the dispersed droplet surface. Tween 20, 40, and 80 were tied as stabilizer in the preliminary trials. Eudragit RS-100 and STP were dissolved in an organic solvent blend consisting of methanol, acetone and dichloromethane. The resultant solution was emulsified with aqueous medium containing a stabilizer while

stirring at 600 rpm. The emulsion was then stirred at room temperature for 3 to 5 hours for solvent evaporation. The collected microspheres were washed 3 times with demineralized water (~ 400 ml). The final product was stored in a vacuum dessicator at 2 to 8^0C. Parameters for all the preparations are summarized in Table 1. The prepared microspheres were evaluated for percentage yield, encapsulation efficiency, particle size, in vitro drug release and anti-inflammatory activity.

Table1 Processing parameters used through the study

Eudragit RS-100	Drug	Continuous Phase	Dispersed Phase, ml Me: Ac : DCM	Stirring Speed	Stabilizer Concentration, ml
300 mg	100 mg	100 ml Aqueous medium	3.5: 5.0: 2.5 3.5: 5.0: 2.5 3.5: 5.0: 2.5	600 ± 10	1, 1.5 and 2

Me, methanol: Ac, acetone: DCM, dichloromethane.

Percentage yield

The percentage yield of microspheres was determined from the ratio of the weight of solidified microspheres obtained to the weight of solid materials used in the inner phase

$$\text{Percentage yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

Encapsulation efficiency

20 mg of microspheres were accurately weighed and crushed by using mortar and pestle. Crushed microspheres were suspended in 10 ml methanol and stirred for half an hour. Then the suspension was filtered through Whatman filter paper No. 44. Then 1 ml of this solution was diluted to 100 ml with distilled water and absorbance was measured at 280 nm against distilled water as a blank. The drug content was determined from the standard curve. Encapsulation efficiency was calculated from following relationship.

$$\text{Encapsulation efficiency} = \frac{\text{Estimated drug content}}{\text{Theoretical drug content}} \times 100$$

Particle size analysis of microspheres

Average particle diameter and size distribution of microspheres were determined by laser diffractometer using a Mastersizer Micro Version 2.19 (Malvern Instruments, Malvern,UK) Approximately 10 mg of microspheres were stirred in 10 ml distilled water. Then aliquot of the microspheres suspension was added into recirculation unit, which was subsequently

circulated 3500 times per minute. Particle size was expressed as equivalent volume diameter. The particle size distribution was also expressed in terms of SPAM factor determined as

$$SPAM = \frac{d_{90} - d_{50}}{d_{10}}$$

Where d_{10} , d_{50} and d_{90} are the diameter sizes and the given percentage value is the percentage of particles smaller that that size. High SPAM values indicate a wide size distribution.

In vitro drug release study of serratiopeptidase microspheres

Microspheres equivalent to 500 mg of Serratiopeptidase were filled in a capsule and in vitro drug release was studied using USP Apparatus II with 900 ml of dissolution medium at 37.5 ± 0.1^0C for 12 hrs at 100 rpm. 0.1 N HCl (pH 1.2) was used as dissolution medium. 10 ml of sample was withdrawn after every hour, and was replaced with an equal volume of fresh dissolution medium. Collector sample were analyzed at 280 nm by spectrophotometrically. The study was performed in triplicate. Dissolution study was also conducted for marketed capsule dissolution data for different formulations of microspheres are reported in Table 3.

Anti-inflammatory activity of serratiopeptidase microspheres

Serratiopeptidase microspheres were evaluated for anti-inflammatory activity by carrageenan induced rat paw oedema method (14,15). Male Albino Wistar rats (150–200 g) were fasted overnight then randomly distributed into 3 groups of 6 animals each. First group served as a vehicle control, second group served as the standard (received Serratiopeptidase 10mg/kg, oraly), while the third test group (received 10 mg/kg, body weight of Serratiopeptidase microspheres) respectively. After 60 min 0.05 ml of 1% w/v suspension of carrageenan was injected into the sub plantar region of right hind paw to all three groups. The rat paw volumes up to lateral malleolar process were measured using Plethysmometer (Basile 7140, Italy) every hour till 4 hours after carrageenan injection, and mean increase in paw volumes were noted. Thus edema volumes in control (Vc) and in groups treated with test compounds (Vt) were calculated. The percentage inhibitions were calculated by using the formula.

$$\%Inhibition = \frac{Vc-Vt}{Vc} \times 100$$

Where, Vc = Edema volume of Control,
Vt = Edema volume of Test

Statistical analysis

The statistical analysis of all the results was carried out using one-way ANOVA followed by Dunnet's multiple comparisons test and all the results obtained in the study were compared

with the vehicle control group. **p < 0.01 were considered statistically significant.

RESULT

Table2 Evaluation of different formulation of serratiopeptidase (SRS) microspheres

Formulation Code	Evaluation parameters		
	% Yield	% Encapsulation Efficiency	Particle Size (µm)
SRS1	82.26	82.862±0.548	32.37
SRS2	92.24	83.461±0.403	32.13
SRS3	83.55	86.427±1.236	31.40
SRS4	87.96	89.758±0.619	31.37
SRS5	92.72	90.982±0.645	31.25
SRS6	85.89	91.407±0.528	31.17
SRS7	90.25	92.625±0.765	32.07
SRS8	96.59	93.827±1.380	33.77
SRS9	85.03	95.424±0.636	33.97
SRL1	86.66	76.16±0.509	34.88
SRL2	90.00	78.72±0.858	32.75
SRL3	84.52	81.75±0.502	33.55
SRL4	86.91	82.31±0.657	31.11
SRL5	87.96	86.70±0.799	30.63
SRL6	88.4	88.26±0.758	31.87
SRL7	89.35	91.42±1.009	32.51
SRL8	96.43	92.56±0.825	31.74
SRL9	85.60	93.60±0.848	30.92

Table 3 *In-vitro* drug release study for serratiopeptidase microspheres

Time (hr)	Cumulative % drug release (mean ± SD., n=3)								
	RS1	RS2	RS3	RS4	RS5	RS6	RS7	RS8	RS9
1	39.17 ±2.14	28.62 ± 1.04	23.48 ± 1.36	21.12 ± 1.50	24.12 ± 0.98	26.69 ± 1.22	25.44 ± 2.12	30.58 ± 0.68	26.21 ± 1.23
2	49.97 ± 1.62	34.11 ± 1.12	26.43 ± 1.53	23.51 ± 1.30	34.74 ± 1.22	36.88 ± 1.77	33.96 ± 1.71	37.41 ± 1.36	30.61 ± 1.21
3	60.94 ± 1.29	43.12 ± 1.28	35.77 ± 2.68	39.78 ± 1.46	43.32 ± 0.95	44.22 ± 1.33	45.33 ± 1.22	48.27 ± 0.79	37.63 ± 1.78
4	64.12 ± 1.50	55.50 ± 0.71	42.80 ± 0.60	48.95 ± 2.17	50.35 ± 0.85	50.68 ± 1.55	52.99 ± 0.44	54.91 ± 2.79	44.84 ± 1.30
5	71.35 ± 1.55	59.47 ± 2.07	47.84 ± 1.56	56.71 ± 1.08	59.49 ± 1.28	56.08 ± 1.72	58.88 ± 1.72	62.42 ± 1.90	51.33 ± 1.78

6	76.66 ± 1.55	64.87 ± 1.02	55.72 ± 1.92	66.49 ± 0.81	67.84 ± 0.99	64.30 ± 0.88	66.45 ± 0.70	66.10 ± 1.76	59.74 ± 2.30
7	85.23 ± 0.81	70.17 ± 0.79	63.33 ± 2.16	72.63 ± 0.92	74.59 ± 1.01	72.43 ± 0.77	71.60 ± 1.70	71.09 ± 1.31	66.54 ± 2.71
8	93.01 ± 1.08	78.59 ± 0.79	70.92 ± 1.62	78.71 ± 1.67	82.51 ± 0.88	79.65 ± 0.56	80.13 ± 2.19	75.94 ± 0.78	71.51 ± 1.06
9	96.87 ± 0.37	85.97 ± 1.05	75.44 ± 0.80	86.21 ± 0.57	88.40 ± 1.32	83.045 ± 0.67	84.50 ± 1.30	83.91 ± 1.64	78.85 ± 2.14
10	100.54 ± 0.45	91.37 ± 1.74	84.97 ± 1.86	90.42 ± 1.11	93.67 ± 1.33	89.30 ± 0.40	90.70 ± 2.22	88.90 ± 2.30	86.53 ± 1.72
11	-------	95.62 ± 0.27	90.84 ± 1.80	95.73 ± 0.37	96.54 ± 1.44	94.13 ± 0.27	95.55 ± 1.11	95.16 ± 0.93	91.05 ± 0.93
12	------	100.26 ± 0.52	95.86 ± 0.54	97.83 ± 0.22	99.00 ± 0.36	97.45 ± 0.11	98.92 ± 0.11	100.27 ± 0.24	95.55 ± 1.12

Table 4 Anti-inflammatory activity of Serratopeptidase on carrageenan induced paw edema in rats (mean ± S.E.M.)

Treatment	**Dose**	**Mean Paw Volume (ml) ± S.E.M**									
		0 hr		**1 hr**		**2 hrs**		**3 hrs**		**4 hrs**	
		EV (ml)	**EI (%)**	**EV (ml)**	**EI (%)**	**EV (ml)**	**EI (%)**	**EV (ml)**	**EI (%)**	**EV (ml)**	**EI (%)**
Vehicle **control**	10 ml/kg	0.93±0.019		1.48±0.030		1.63±0.061		1.76±0.083		1.72±0.041	
Serratiopeptidase **(Standard)**	10 mg/kg	0.98±0.050	-5.37	1.14±0.016**	22.97	1.33±0.113	18.4	1.48±0.104	15.9	1.22±0.051**	29.06
Serratiopeptidase microsphers **(Test)**	10 mg/kg	1.03±0.037	-10.75	1.11±0.080**	25.0	1.07±0.077**	34.35	1.16±0.085**	34.9	1.06±0.092**	38.37

Vehicle control received distilled water **p<0.01*

Values are Mean Paw Volume (ml) ± S.E.M (n=6)

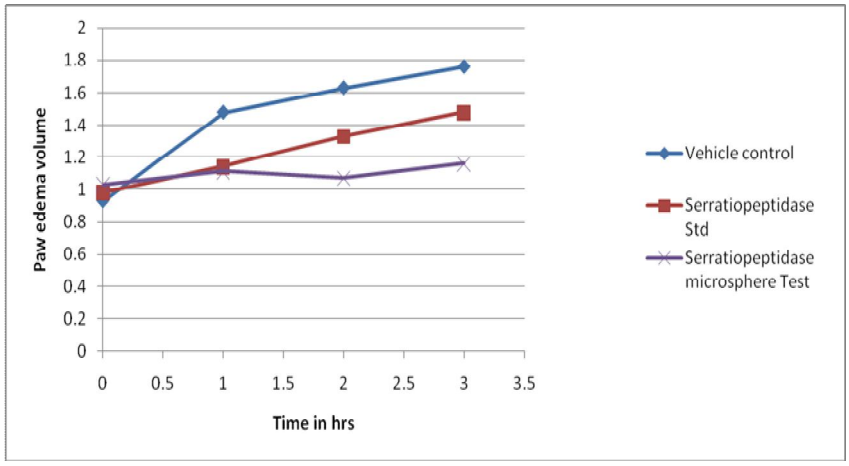

Fig 1 Anti-inflammatory activity of Serratiopeptidase on carrageenan induced paw edema

DISCUSSION

Emulsion Solvent Evaporation technique was chosen because good results of percent drug encapsulation found by this technique.

Effect on drug encapsulation efficiency

The emulsion solvent evaporation method showed good encapsulation efficiency. Percent drug encapsulated was found to be in a range of 82-95% for Eudragit RS100 microspheres. It was observed that with increase in polymer concentration drug encapsulation efficiency was increased (Table 2). Drug encapsulation efficiency of Eudragit RS100 was slightly increased as the Tween 80 concentration were increased; because dispersing agent decrease the interfacial tension between the lipophilic and hydrophilic phases of the emulsion and simplify the formation of microspheres also this dispersing agent provides a thin protective layer around the droplets and reduces the extent of their collision and coalescence.

Effect on particle size

Particle size for Eudragit RS100 microsphere was found in the range of 31.17-33.97 µm with SPAN factors ranging between 1.24-1.64 (Table 2). In all batches of Serratiopeptidase microspheres it was observed that the size of microspheres increased as the concentration of inner phase polymer was increased while the concentration of dispersing agent was kept constant. This can be approved to an increased in the viscosity of internal phase caused coalescence of inner phase droplets. But the variations of the concentrations of Tween 80 did not affect the particle size of microspheres. SPAN factors for all the batches ranges in between 1.20-1.64, which indicates narrow size of distribution.

Effect on drug release

In vitro dissolution results showed that the microspheres prepared with a different core-coat ratio gave better-sustained action (Table 3). Eudragit RS100 showed sustained action over 12 hrs Table 3 clearly illustrates that the rate of drug release from the microspheres depended on the polymer concentration of the prepared devices. An inverse relationship was observed between polymer content and drug release rate from the prepared microspheres. In all cases of polymers it was seen that microspheres containing 10% polymer released the drug more rapidly, while those with 20% polymers exhibited a relatively slower drug release profile.

Anti-inflammatory activity

Carrageenan induced paw oedema was taken as a prototype of exudative phase of acute inflammation. Inflammatory stimuli microbes, chemicals and necrosed cells activate the different mediator through a common trigger mechanism. Carrageenan oedema consists of three distinct phases; an initial release of histamine and serotonin (5-Hydroxy tryptamine), a second phase mediated by kinins and finally a third phase, the mediator of which is suspected to be prostaglandin and leucotrienes (16-19). Most of the NSAIDs have well-balanced anti-inflammatory and ulcerogenic activities, which are considered to be due to PG synthetase inhibitor activity.

The data obtained from the present study indicate that microspheres of Serratiopeptidase produced anti-inflammatory effect on carrageenan-induced paw oedema (Table 4). The effect of the Serratiopeptidase was most pronounced at the later stages of the inflammatory response, which corresponds to the phase of prostaglandin as well as leukotriene release.

The liberation of free arachidonic acid from damaged cell membranes catalyzed by phospholipase A2 and the further metabolites of arachidonic acid catalyzed by cyclo-oxygenases and lipoxygenases generate a range of important inflammatory mediators. Prostaglandins, such as prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2) are potent dilators of vascular smooth muscle, accounting for the characteristic vasodilatation and erythema (redness) in acute inflammation (20 Solomon et al., 1968). PGE2 also acts synergistically with other mediators to produce inflammatory pain. The products of 5-lipoxygenase including leukotrienes (LTs) constitute an important class of inflammatory mediators of asthma and various inflammatory diseases (21). There is also some evidence that lipoxygenase products contribute to vascular changes in inflammation (22). Inhibition of

the biosynthesis of inflammatory mediators by blocking the activities of those enzymes would be the possible mechanism of action of serratiopeptidase.

Comparatively proteolytic activity of intestinal mucosa is less than that observed in the stomach. Intestinal specific drug delivery system protects peptide drugs from hydrolysis and enzymatic degradation in the stomach and eventually releases drugs in the jejunum, duodenum, ileum or colon which promises greater systemic bioavailability (23).

CONCLUSION

This research work provided a novel simple approach to formulate microspheres of serratiopeptidase enzyme. The controlled release microspheres were successfully formulated using Eudragit RS 100 by emulsion solvent evaporation technique for delivery of drug over extended period of time. In vitro pharmacokinetic study proved that serratiopeptidase from the microspheres shows prolong release and may be able to sustain the therapeutic effect. It can be concluded that the serratiopeptidase microspheres possesses anti-inflammatory properties, which is probably mediated via inhibition of prostaglandin synthesis as well as leukotriene synthesis. The serratiopeptidase will be beneficial in the management of pain and inflammatory disorders.

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