ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

p^H-Responsive and Mucoadherent Microparticles for Oral Delivery of Serratiopeptidase: Development and In Vitro Characterization

Preeti K Suresh[1]* and Anish Chandy[2]

[1] University Institute of Pharmacy, Pt. Ravishankar Shukla University, Raipur (CG) INDIA
[2] School of Pharmacy, Chouksey Engineering College, Bilaspur (CG) INDIA

ABSTRACT

The aim of the present study was to explore the potential of enteric coated chitosan microspheres as carriers for oral site-specific delivery of peptides and proteins. In this study, microspheres of serratiopeptidase were prepared by water in oil (w/o) emulsification solvent evaporation technique. Ratio of chitosan and serratiopeptidase was varied in the range of 1:1, 1:2 and 2:1. The microspheres were then coated with Eudragit L 100. The product was obtained as non-aggregated free flowing microparticles. The drug content was found to be between 15 to 40%. The presence of protein drug in the microparticles was confirmed by SDS-PAGE technique. In vitro drug release studies indicated pH-responsive release profile. In vitro release of the drug first into phosphate buffer pH 1.2 for 1 hr and then in phosphate buffer pH 6.8 at 37ºC was determined as a function of crosslinking coating density of the microparticle. The pH-responsive behavior of the microparticle was confirmed, as there was less than 10% release in phosphate buffer pH1.2. The extent of drug release had a remarkable dependence on the coating density of the Eudragit L 100, the highly crosslinked spheres releasing only around 35% of the incorporated drug in 6 hour compared to 40% from lightly coated spheres. The mucoadhesive nature of the microparticles was established by an in situ method. These results indicate that Eudragit L 100 coated chitosan microparticles are promising carriers for pH-responsive oral drug delivery of peptides and proteins.

Keywords: peptide, Eudragit L 100, Chitosan, pH-dependent, pH-sensitive, intestine-specific.

Corresponding author
Email: preeti_venugopalan@yahoo.co.in

ISSN: 0975-8585

INTRODUCTION

The intestinal drug targeting has been exploited for systemic delivery of active drugs. Most of the peptide and protein drugs are unstable in the stomach and upper part of intestine. Apart from stability problems, peptides are not well absorbed from the lumen of the GIT due to their large molecular size and have high sensitivity to brush border peptidase activity. Comparatively, proteolytic activity of intestinal mucosa is less than that observed in the stomach. Intestinal specific drug delivery systems protect peptide drugs from hydrolysis and enzymatic degradation in the stomach, and eventually release drugs in the jejunum, deuodenum, ileum or colon, which promises greater systemic bioavailability.

Serratiopeptidase (STP) is an anti-inflammatory, proteolytic enzyme isolated from the microorganism, Serratia sp. HY-6 and is orally active [3]. Oral proteolytic enzymes have been used successfully for inflammatory conditions. Recently, the intestinal absorption of orally administered STP has also been demonstrated. To achieve an ideal therapeutic effect, however, it is essential that any enzyme preparation be enterically coated so as to release the enzymes in the intestines (where they can be absorbed) and not in the stomach (where they can be digested). STP degrades in the stomach, but it is well absorbed from the small intestine.

Microsphere based drug delivery systems have received considerable attention in recent years. The most important characteristic of microspheres is the microphase separation morphology, which endows it with a controllable variability in degradation rate and drug release.

pH-activated drug delivery systems permit the drug release at specific pH and the drug molecules can thus be protected from degradation in the hostile gastric environment and in the intestinal fluid the polymers dissolve and release the drug at intestinal pH>6.8. These types of systems can be fabricated with polymers which are insoluble in acidic pH of stomach, but soluble in basic pH prevailing in the intestine, viz., Eudragit L100, Eudragit S, Eudragit L, HMCP, etc.

The aim of the present of work was to achieve site-specific controlled delivery of STP in the small intestine. To achieve this objective Eudragit coated chitosan microparticles of STP were prepared. The Eudragit coating will dissolve in the small intestine leaving chitosan microsphere, which swells in the intestinal fluid leading to controlled release of STP from the microspheres. The proposed mechanism is illustrated in Figure 1.

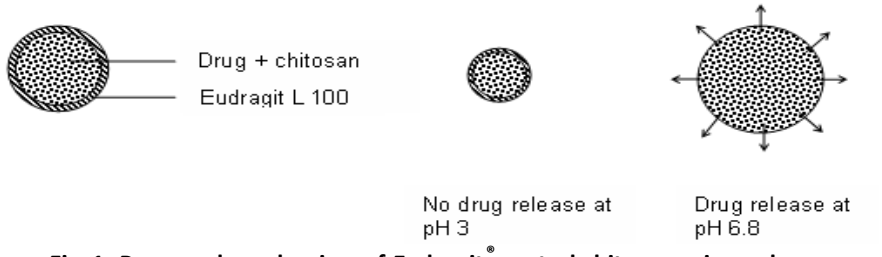

Fig. 1: Proposed mechanism of Eudragit® coated chitosan microspheres

ISSN: 0975-8585

MATERIAL AND METHODS

Materials

Chitosan (degree of deacetylation, 85.2%) particles with a viscosity 50 cps was obtained as a gift sample from CFTI, Kochi, India. Serratiopeptidase was obtained from Centaur Labs Ltd, Mumbai, India, as a gift sample. All other reagents used were of analytical grade from different commercial manufacturers.

Development of Microparticles

Preparation of STP-containing chitosan microspheres (Chi-STP)

Chitosan (Chi) microspheres containing STP were prepared using w/o emulsification–solvent evaporation method as reported by Hori et al [2]. Chitosan (100 mg) was dissolved in 5 ml of a 1% (v/v) acetic acid aqueous solution, and to this 2 ml of aqueous solution containing STP (100 mg) was added. The chitosan (Chi) solution containing STP was dripped over 5 min into 200 ml of liquid paraffin containing SO-15® at 1% (w/v), which was continuously stirred at 500 rpm. The w/o emulsion obtained was stirred vigorously at 35 C for 24 h, diethyl ether was added, and the mixture was further, stirred overnight. The particles obtained were washed with diethyl ether, 25% (w/w) ammonia aqueous solution, ethanol and diethyl ether in that order. The particles were obtained by filtration and dried in air to yield microspheres.

ER-coated Chi-STP microspheres

Eudragit® (ER)-coated Chi-STP microspheres (ER-Ch-STP) were prepared by coating Chi-STP with Eudragit L 100 following the method reported by Bogataj et al [1]. Eudragit L 100 was dissolved in 20 ml of acetone and the chitosan microspheres were dispersed in it. Then equivalent amount of magnesium stearate was added and the dispersion was added dropwise to 100 ml liquid paraffin (Heavy: Light = 1:1). To this dispersion 20 ml hexane was added with stirring. The mixture was stirred for 90 min and the microspheres formed were collected by filtration and washed with hexane twice and dried at room temperature.

Optimization of Process Variables

Various process variables, which could affect the preparation and properties of the microspheres, were identified and studied. Preparation of pH-responsive microspheres involves various process variables out of which drug: polymer ratio (D), temperature (T) and coating polymer: microsphere ratio (P) was selected for the optimization of formulation.

The formulation code and respective variables used in the preparation of microspheres are listed in Table 1. The effect of these variables was observed on particle size and size distribution, % drug entrapment and % yield of microspheres.

ISSN: 0975-8585

Table 1: The composition, formulation code and variables used in the preparation of microspheres

Formulation code	Drug: Polymer ratio	Temperature (°C)	Microsphere: Coating Polymer ratio
A_1	1:1	35	1:1
A_2	1:1	35	1:2
A_3	1:1	35	1:5
B_1	1:1	40	1:1
B_2	1:1	40	1:2
B_3	1:1	40	1:5
C_1	1:1	45	1:1
C_2	1:1	45	1:2
C_3	1:1	45	1:5
D_1	1:2	35	1:1
D_2	1:2	35	1:2
D_3	1:2	35	1:5
E_1	1:2	40	1:1
E_2	1:2	40	1:2
E_3	1:2	40	1:5
F_1	1:2	45	1:1
F_2	1:2	45	1:2
F_3	1:2	45	1:5
G_1	2:1	35	1:1
G_2	2:1	35	1:2
G_3	2:1	35	1:5
H_1	2:1	40	1:1
H_2	2:1	40	1:2
H_3	2:1	40	1:5
I_1	2:1	45	1:1
I_2	2:1	45	1:2
I_3	2:1	45	1:5

CHARACTERIZATION

Size distribution and morphology

Scanning Electron Microscopy (SEM JEOL JSM-5800, Japan) was used to evaluate the surface texture, shape and size of the microspheres. The samples for SEM were prepared by lightly sprinkling the microspheres powder on a double adhesive tape, which stuck to an aluminum stub. The stubs were then coated with gold to a thickness of about 300 A using a sputter container. The photomicrographs are shown in Figures 2 and 3.

ISSN: 0975-8585

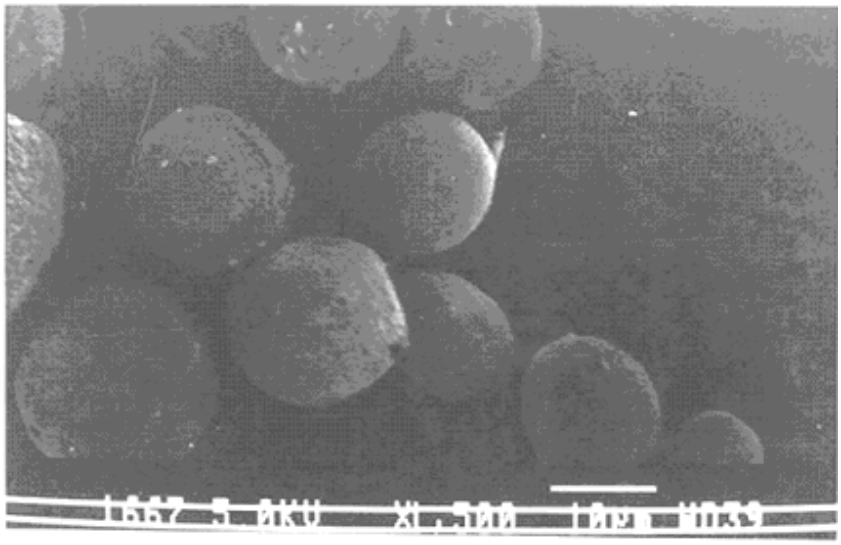

Fig. 2: Scanning electron microscopy of uncoated microspheres (D)

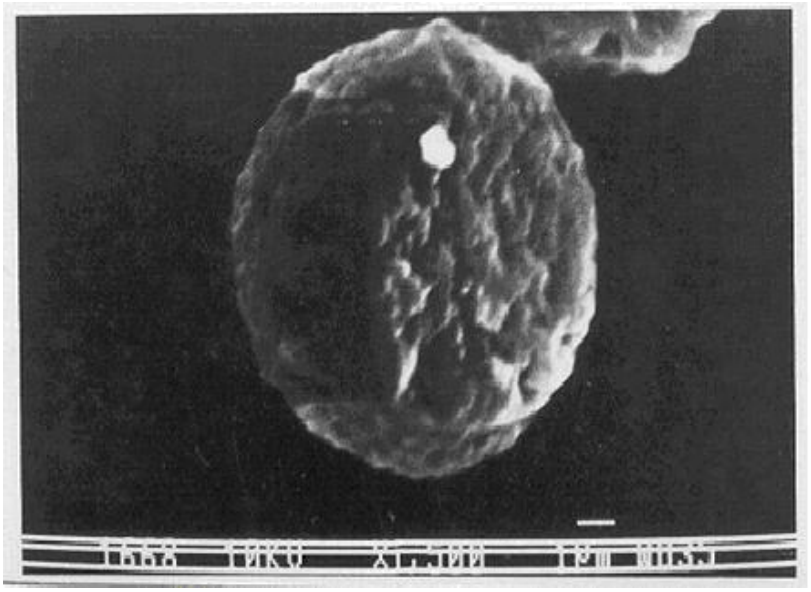

Fig. 3: Scanning electron microscopy of coated microspheres (D_1)

Yield

To determine the % yield, weight of prepared microspheres was divided by the total weight of all the non-volatile components used for the preparation of the microspheres. The results are presented in Table 2.

Drug content

Chi-STP microspheres (10 mg) were dissolved in 10 ml of SIF (pH 7.4) by stirring with a vortex mixer. An aliquot of 150 µl of the solution was treated with casein solution to detect its

proteolytic activity and to measure the amount of STP from the standard curve. The resultant solution was centrifuged at 3000 rpm for 5 min and the supernatant was analyzed spectrophotometrically at 280 nm.

Table 2: Encapsulation efficiency, yield, drug content, particle size and coating thickness of prepared microparticles

Formulation	Encapsulation efficiency (%)	Yield (%)	Drug content (%)	Particle size (µm)	Coating Thickness (µm)
A1	99.7	99.65	16.15	13.69	6.89
A2	98.1	98.41	10.73	15.86	9.06
A3	109.2	95.49	5.32	17.08	10.28
B1	99.5	99.58	11.54	14.32	6.22
B2	100.2	98.02	7.34	15.68	7.58
B3	101.5	95.13	3.68	18.89	10.79
C1	99.7	99.01	10.14	13.78	6.28
C2	98.3	99.50	6.76	15.78	8.28
C3	98.9	93.41	3.38	17.75	10.75
D1	101.2	99.31	21.15	14.86	6.56
D2	98.5	99.62	14.12	16.38	8.08
D3	99.8	98.39	7.05	20.46	12.16
E1	103.2	99.32	15.77	13.70	6.40
E2	99.6	98.56	10.05	15.72	8.42
E3	99.3	96.21	5.25	17.80	10.50
F1	99.6	99.45	14.23	13.76	6.86
F2	99.9	98.92	9.51	15.90	9.00
F3	99.4	96.48	4.72	17.65	10.75
G1	101.4	98.48	10.81	13.28	6.58
G2	99.5	99.70	7.22	15.31	8.61
G3	99.6	96.52	3.62	18.08	11.38
H1	99.6	99.61	7.56	14.56	6.45
H2	99.9	98.32	5.04	15.25	7.14
H3	99.3	95.46	2.52	18.60	10.50
I1	99.2	99.31	7.53	16.70	8.00
I2	99.6	99.05	5.02	18.48	9.78
I3	99.5	94.68	2.51	26.65	17.95

In vitro release profile

Accurately weighed (60 mg) microspheres equivalent to 10 mg drug were suspended in 500 ml of mixed phosphate buffered saline (pH 6.8), and incubated at 37^{o}C and 90 rpm. After 1, 2, 3, 4, 5, 6 and 24 h incubation, 1ml of medium was collected, 4.5 ml Casein solution added and allowed to stand for 37^{o}C for 20 min. [5] To the above solution, and trichloroacetic acid was added to break the reaction. The supernatant collected and was used for the quantitative assay of STP. For this assay, the final sample was obtained after centrifugation at 10,000 rpm for 5 min, and absorbance was measured spectrophotometrically at 280 nm.

ISSN: 0975-8585

ER-Chi-STP accurately weighed microspheres equivalent to 10mg drug were suspended in 500 ml of phosphate buffered saline (pH 1.2) and incubated at 37°C at 90 rpm. The samples were collected at regular intervals of 15 min. The pH was then adjusted to pH 6.8. At 1, 2, 3, 4, 5, 6 and 24 h after the start of the incubation in mixed phosphate buffered saline (pH 6.8), 1ml of the medium was taken, 4.5 ml casein solution was added and allowed to stand at 37°C for 20 min. Trichloroacetic acid was added to this solution to break the reaction and then centrifuged at 10000 rpm for 10 min. The supernatant was used for the measurement of the amount of STP released. Proteolytic activity was determined to measure the concentration of STP by spectrophotometric estimation at 280 nm. The results are presented in Figures 4-6.

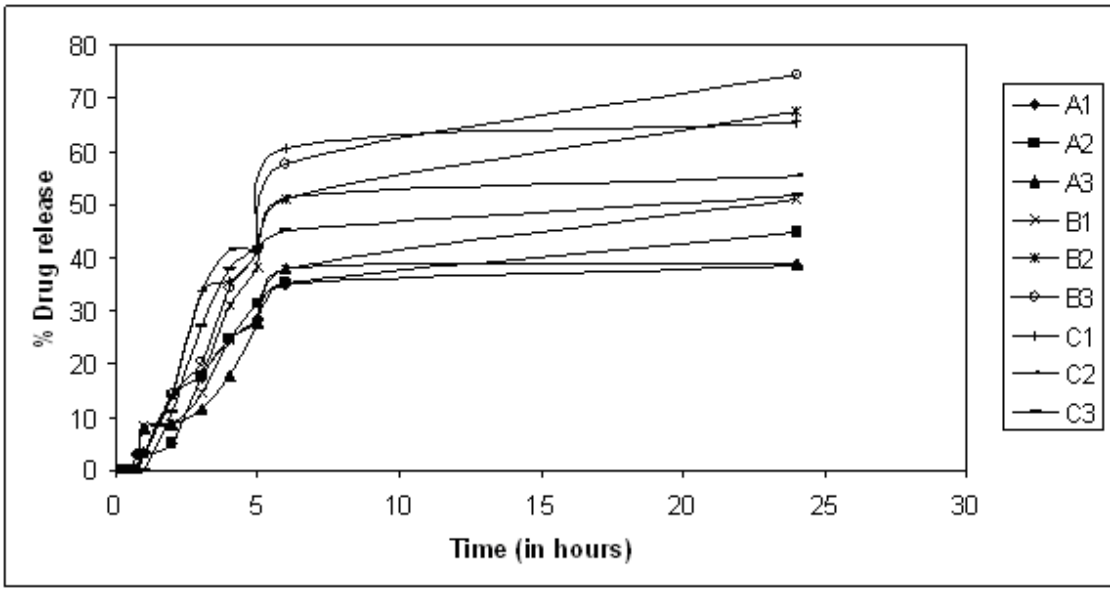

Fig. 4: Drug release profile of various microparticles (drug: polymer ratio of 1:1)

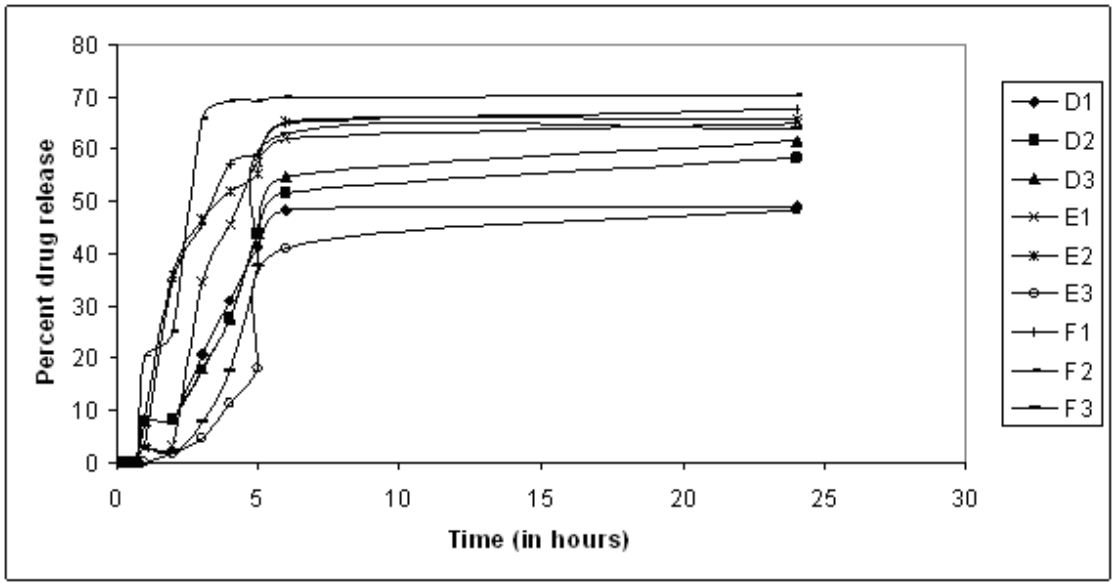

Fig. 5: Drug release profile of various microparticles (drug: polymer ratio of 1:2)

ISSN: 0975-8585

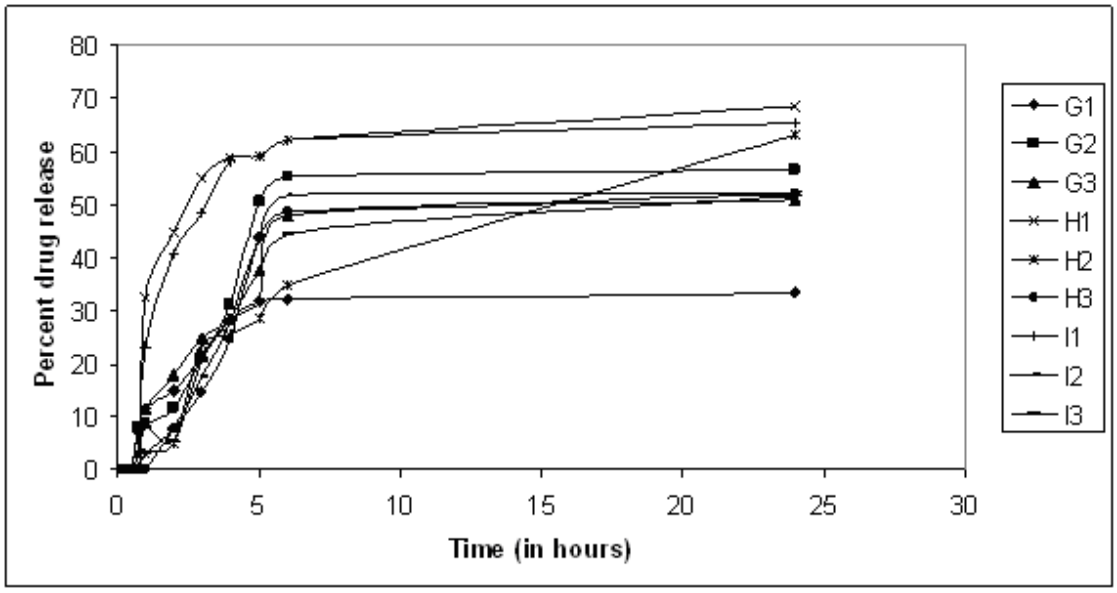

Fig. 6: Drug release profile of various microparticles (drug: polymer ratio of 2:1)

Detection of Drug by SDS PAGE

10 mg microspheres were dissolved in 1ml phosphate buffered saline (pH 7.4), then vortexed for 10 min, allowed to stand for 5 min and again vortexed for 5 min to obtain equilibrium concentration of drug in solution. The sample was centrifuged at 10,000 rpm for 10 min. Supernatant was collected and used for SDS- PAGE. 20µl of supernatant was taken and applied on gel with reducing buffer and dye methylene blue. The stain was detected by marker silver stain. This procedure was repeated for non-reducing condition except using non-reducing buffer in place of reducing buffer (Figure 7).

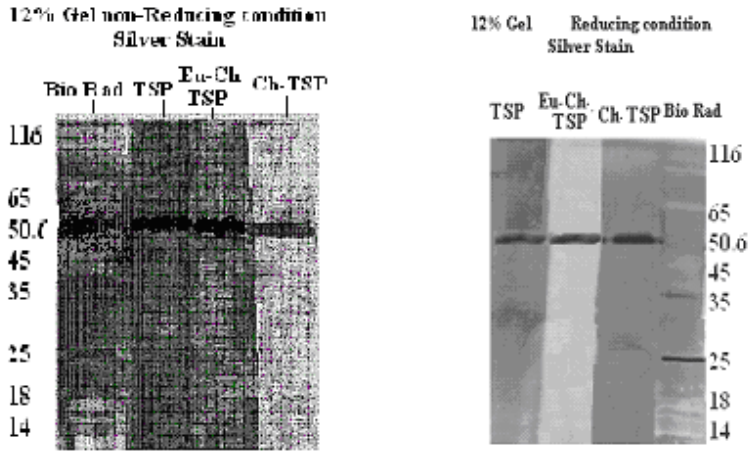

Fig. 7: SDS PAGE analysis of Serratiopeptidase in microspheres

ISSN: 0975-8585

Zeta potential

The microspheres were suspended in Phosphate buffer (pH 1.2) for 30 minutes. The suspension (2% w/v) was employed for the determination of zeta potential. The results are presented in Table 3.

Table 3: Zeta potential of selected microsphere formulations

Formulation	Zeta potential (V)
A_1	+ 13.2
D_2	+ 8.5
Void Microspheres	+ 40.0

Mucoadhesivity

Mucoadhesivity was determined by an in situ method as described by [4]. A freshly cut 5-6 cm long piece of small intestine of rat was obtained and cleaned by washing with isotonic saline. The piece was reversed with steel rod and the mucosal surface was exposed. Known weights of microspheres were suspended in test tube containing phosphate buffer (pH 7.4) and intestine section (exposed mucosal surface) was introduced into the suspension with a thread. The intestinal piece was maintained at 80% relative humidity for 30 min in a desicator at 37 C. The piece was taken out and phosphate buffer pH 6.8 was allowed to flow over the intestinal piece for about 2 minutes at a rate of 20 ml/min. The perfusate was collected and dried to recover the unadhered particles. The % mucoadhesion was estimated by the ratio of amount applied to adhered microspheres. The results are presented in Table 4.

Table 4: Percent mucoadhesion of various formulation of microspheres

Formulation	% Mucoadhesion
A_1	36.95 ± 3.66
G_2	40.13 ± 0.10
D_1	19.16 ± 5.85
D_2	31.66 ± 1.66

Swelling properties

The water uptake by the non-crosslinked and crosslinked chitosan microspheres was measured gravimetrically by swelling microspheres in deionized water (pH 6.0, 37°C) and measuring changes in their weight during swelling. Microspheres were weighed and placed inside a dialysis membrane, which was then introduced into the medium (50 ml) under continuous stirring at 50 rpm and allowed to swell. The swollen samples were removed after 120 min and their surfaces were blotted with a filter paper to remove medium adsorbed on the surface and then immediately weighed. Each swelling experiment was repeated twice and the average value was taken as the degree of swelling, as calculated by:

ISSN: 0975-8585

$$S.W. = M_t - M_o / t$$

where, M_t denotes the weight of the swollen sample at time t and M_o is the initial weight of the sample before swelling. The results are presented in Table 5.

Table 5: Degree of swelling of coated formulation

Formulation	Degree of swelling
A_1	0.76 ± 0.04
A_2	0.86 ± 0.04
A_3	0.80 ± 0.02
E_1	0.78 ± 0.04
E_2	0.72 ± 0.06
E_3	0.78 ± 0.04
G_1	0.80 ± 0.03
G_2	0.78 ± 0.02
G_3	0.66 ± 0.06

X-Ray Powder Diffractometry

X-ray powder diffractometry (XRD) was carried out to investigate the effect of microencapsulation process on crystallinity of the drug. Powder XRD patterns were recorded on a powder XRD using Ni-filtered, CuKα radiation, a voltage of 30 Kv, and a current of 15 mA. The scanning rate employed was 1° min^{-1} over the 20° to 60° diffraction angle (2θ) range. The XRD patterns of polymers, drug and drug-loaded microspheres were recorded (Figure 8). Microspheres were triturated to get fine powder before taking the scan.

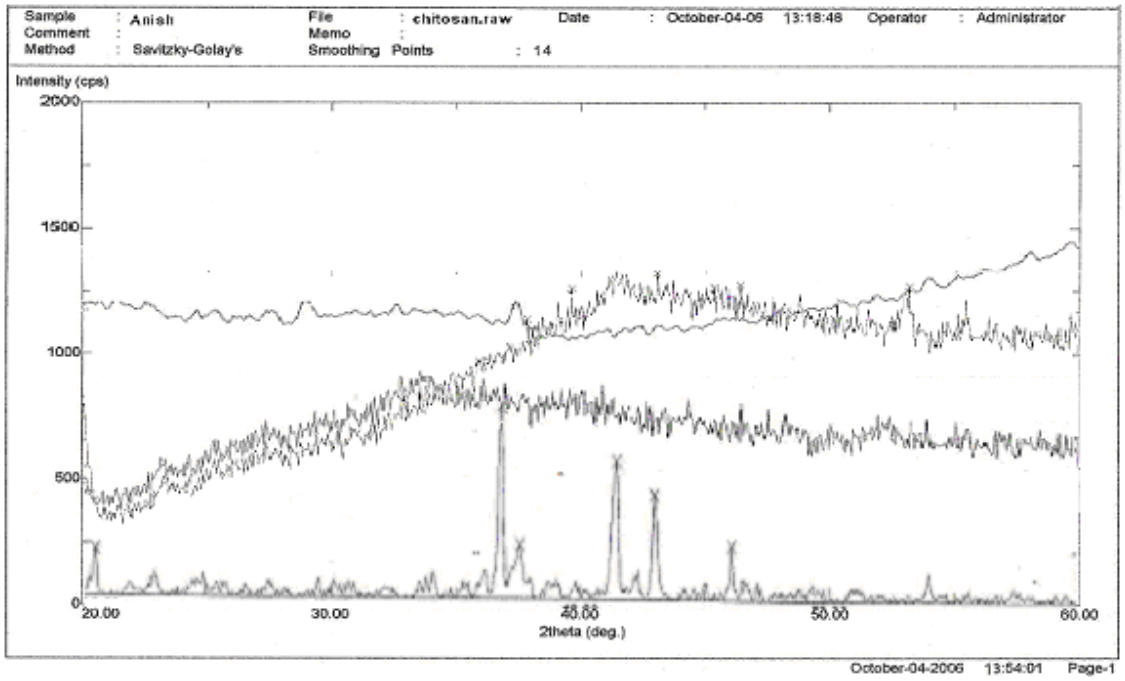

Fig. 8: X-ray powder diffractometry of serratiopeptidase, polymer, chitosan and microparticles.

ISSN: 0975-8585

Statistical Analysis

Statistical significance of all the data generated was tested by analysis of variance (ANOVA) followed by studentized range test. A confidence limit of $P<0.05$ was fixed for interpretation of the results using the software PRISM (Graphpad, San Diego, CA).

RESULTS AND DISCUSSION

Some of the challenges of encapsulating proteins/enzymes for sustained delivery namely high loading with high recovery, maintenance of enzyme integrity and sustained delivery with a pH-responsive release have been successfully met with microspheres in this study. It offers advantage for drug delivery of proteins/ enzymes, which can also be extended to other therapeutic macromolecules.

Preparation of the microparticles

Chi-STP microspheres prepared by emulsification–solvent evaporation technique showed good granulation. The particle shape was nearly spherical (Fig. 2). The mean particle diameter was 8.3 m. The mean STP content was between 13.731.2% (w/w), and the encapsulation efficiency was more than 38%. ER-Chi-STP was prepared at different combination ratios of Chi-STP to ER. The drug content was almost proportional to the ratio of Chi-STP. Encapsulation efficiencies were 95.3–119.2%, indicating that the ideal drug content was well achieved. Coated microspheres prepared by simple emulsification solvent evaporation method with Chi-STP microspheres and Eudragit, showed a larger size, with the increase in amount of Eudragit. The particle shape of A_3, B_3, etc. was irregular, but rugged ellipsoid.

Morphology of microspheres was examined by optical and scanning electron microscopy. The view of the uncoated microspheres showed a hollow structure with a smooth surface morphology whereas coated microspheres displayed a rough surface. The shell of the uncoated microspheres showed some porous structures, which might have been caused by the evaporation of solvent entrapped within the shell of microspheres after forming a smooth dense skin layer. In contrast, the coated microspheres displayed continuous surface without any porous structure.

The effect of the ratio of drug: polymer (Chitosan) in the aqueous phase on the formation of the microspheres was evaluated keeping the volume of aqueous phase constant at 5 ml. The yield of the microspheres was found to be 85-98%. A variation in drug release was observed, with highly loaded microspheres releasing more drugs. However, the average particle size and the coating thickness increased as the amount of polymer were increased as reported by Hori et al. [2]

The temperature of the dispersing medium was an important factor in the formation of microspheres, because it controls the evaporation rate of the solvent. The microspheres

prepared at low temperatures (35° C), had irregular morphology. At higher temperatures (45° C), the microspheres prepared had a darker brownish color and uniform shape. The optimum temperature to form good microspheres was found to be 35 and 40° C.

Emulsification–solvent evaporation technique gave Chi-STP microspheres, which had pores adequate for release of STP. The coating with ER prevented Chi-STP from the dissolution in SGF (pH 1.2). ER-coated Chi-STP microspheres released STP very slowly in mixed phosphate buffered saline (pH 6.8), than Chi-STP. It was observed that high entrapment efficiency was achieved at optimum drug: polymer ratio and optimum temperature.

In vitro drug release

Release of drug from pH responsive microspheres was evaluated at pH 1.2, and pH 6.8. The initial slow release at the entire tested medium may be due to coating with Eudragit L100. The uncoated microspheres of chitosan alone released a significant amount of drug through the pores of the microspheres, as the polymer was soluble in acidic pH whereas the coating prevented the release of drug. A combination of polymer was used for current study to design pH responsive delivery system, which released most of the drug at lower gastrointestinal tract. As the amount of Eudragit L 100 used in the preparation was increased, the release of the drug decreased. The release in the mixed phosphate buffer (pH 6.8) may be due to the porous nature of chitosan and Eudragit L100 and increase in the pores of the microspheres due to swelling of chitosan. As the drug was most soluble in pH 6.8 and 7.4, its release was highest in these mediums caused by diffusion through pores. The release of drug in the mixed phosphate buffer (pH 6.8) was many times elevated than at pH 1.2. At pH 6.8, nearly 50% of the drug was released as observed within first 6 hours. It may be due to swelling of chitosan in mixed phosphate buffer.

In case of formulation A_1 coated with Eudragit L 100, the maximum drug release was seen in pH 6.8 and minimum at pH 1.2. There was an initial fast release of drug in pH 6.8, which may be due to the dissolution of Eudragit L 100, in alkaline pH.

Formulation A_3 prepared by coating with polymer at highest ratio to microsphere, also showed the maximum drug release at pH 6.8. The effect of coating ratio of polymer to microsphere on dissolution time was in the sequence as1:1<1:2<1:5, dissolution time.

X-ray powder diffraction study

The x-ray powder diffraction patterns of microspheres along with those of physical mixtures and raw crystals of drug and polymer were studied. The Serratiopeptidase-Chitosan-Eudragit system, in the form of microsphere, indicated the presence of the crystalline STP but with a dramatic decrease of the intensity of the signal because of both a dilution effect and a decrease in crystallinity of the drug. These results were in compliance with the reports of [6].

Detection of drug by SDS-PAGE technique

Formulation A and A_1 was identified and tested for purity by SDS-PAGE technique. The presence of drug in the microsphere was confirmed by this technique.

Bioadhesive study

The bioadhesion of microspheres was measured by the method reported by Rao and Buri [4]. The adsorption of microsphere to mucin is expected to be dominated by the electrostatic attraction between the positively charged chitosan and negatively charged mucin. Therefore, the surface charges of microspheres represented by zeta potential influenced the amount bioadhesed. The amount of microsphere adsorbed increased with increasing chitosan concentration. Also, in conformity with the electrostatic attraction theory, the amount of the adsorption decreased with decreasing zeta potential. Microspheres with highest zeta potential are highly adsorbed. The mucoadhession of the microspheres increased with increase in net concentration of chitosan.

The water uptake of microspheres prepared at 40ºC and 45ºC was more than those prepared at 35ºC. It also increased with increase in chitosan content.

CONCLUSION

The drug carriers developed for oral peptide delivery in the present study have been tailored to protect the sensitive macromolecular drug from the harsh environment of the stomach and deliver biologically active peptide for an extended period of time to more favorable regions for absorption along the GI tract. The results indicated pH-responsive release of serratiopeptidase and also confirmed integrity of drug within the formulation. The integrity of the drug could be maintained during the formulation process and other formulation objectives i.e., high encapsulation yield and loading combined with pH responsive release of drugs were also achieved. Further, the method of preparation of microspheres adopted i.e. the emulsification process, is amenable to easy industrial scale-up. These findings corroborate the idea that the combination of pH-responsive and mucoadhesive properties can be utilized effectively to devise strategies for oral delivery of peptidal drugs.

Declaration of Interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Bogataj M, Burjak M, Velnar M, Grabnar I, Mrhar A. Int J Pharm 2001; 224: 123-30.
[2] Hori M, Onishi H, Machida Y. Int J Pharm 2005; 297: 223-34.

[3] Moriya N, Nakata M, Nakamura M, Takaoka M, Iwasa S, Kato K, Kakinuma A. Biotechnol Appl Biochem 1994; 20: 101-8.
[4] Rao KVR, Buri P. Int J Pharm 1989; 52: 265-70.
[5] Ryden AN, Hofsten BV. Acta Chemica Scandinavica 1968; 22: 2803-8.
[6] Tayade PT, Kale RD. AAPS PharmSci 2004; 6(1): E12.