INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

FEASIBILITY, FORMULATION AND CHARACTERIZATION OF INNOVATIVE MICROPARTICLES FOR ORAL DELIVERY OF PEPTIDE DRUG

Bodhankar MM[1], Agnihotri VV[*1] and Bhushan SB[1]

[1]Department of Pharmaceutics, Gurunanak college of Pharmacy, Nagpur, Maharashtra, India.

*Corresponding Author: vinitagnihotri_05@rediffmail.com

ABSTRACT

Serratiopeptidase (SRP) was use as a model peptide. Microparticles were formulated with phase-separation coacervation method. SRP was entrapped in bovine serum albumin and then enteric coated with Eudragit S-100. The microspheres were characterized for morphology, particle size, encapsulation efficiency, drug release and *in-vitro* Proteolytic activity. The preliminary studies show that albumin and Eudragit were compatible with SRP. Drug release was also studied with colonic caecal contents to access colonic delivery of peptide. These microspheres are able to entrap the peptide at high levels where the inner albumin core containing the drug would allows kinetic control of release and coating ensures drug core localization in intestinal tract. Enteric coated cross linked albumin microspheres can be considered as promising delivery systems for oral delivery of peptide drugs like serratiopeptidase.

Keywords: Serratiopeptidase and Micropartiles.

INTRODUCTION

Oral rout of administration has been most popular from the decade and still preferred by patients and Physicians. Solid dosage forms have inherent advantages because they have a high-metering accuracy, the application of them is very easy and comfortable to formulator, patient and Physicians and their stability is very good. The successful oral administration of therapeutic peptides and proteins remains one of the main challenges for pharmaceutical technologists. The enzymatic degradation in GI tract and low membrane permeation due to hydrophilic characteristics of proteins and peptide attribute to low bioavailability of this therapeutics[1,2].

The unique structural characteristics of amino acid based biopharmaceuticals make the formulation and development more challenging than for more conventional drugs. Firstly, the backbone and folding structure of proteins must be retained during manufacturing consideration and storage. Second and most critical challenge in such drug delivery approach is to preserve the formulation during its passage through the stomach, under degradative conditions, such as the presence of enzymes and about first six meters of the small intestine[3], in order to ensure efficient delivery. Various approaches have been described in the literature to overcome these barriers, including the co-administration of protease inhibitors[4], the

utilization of multifunctional polymers[5] and micro-/nanoparticulate drug delivery systems[6].

One strategy to overcome body's natural process is to alter the environment for maximum solubility and enzyme stability of protein by using formulation excipients such as buffer surfactant and protease inhibitor. If the enzyme attack can be defeated or delayed, the protein can be presented for absorption as well as mean colonic transit time in humans is reported to be as high as 33 h in men and 47 h in women[7], therefore such approach can be utilized for prolong systemic delivery of therapeutic protein and peptides.

In-Vitro release testing is mostly carried out in buffer solutions under shaking, which may strongly influence the peptide stability[8]. During *in-vitro* release, the peptide may also adsorb on the surface of the microparticles, leading to an underestimation of the amount of peptide effectively released. For example, insulin was found to have a higher affinity to the lower molecular weight (40000) DL-PLA than to the higher one (140000)[9]. The degree of adsorption and the formation of peptide: protein multilayer's around biodegradable microparticles are directly related to the hydrophobicity and the concentration of the polymer and the peptide[10, 11, 12]. In a previous paper[13], it has been reported that the onset time of drug release in vitro is delayed depending on the thickness of the acid-soluble polymeric layer. This suggested that the CTDC has a potential for colon-targeted delivery of drugs, when sufficient acid-resistibility and the suitable thickness of coating are given to adjust the onset time of drug release to the small intestinal transit time, which is known to be 3±1 h[14].

The excipients induced instability in protein and peptide drug may follow the physical and chemical degradation of native protein. These incompabilities may consequently induce the change in conformation and structures of proteins. To preserve the formulation from hydrolysis and enzymatic degradation they are mostly formulated with the enteric excipients. As the proteins are very sensitive to their environment, these incompabilities may be provoked when the formulation is subjected dissolution in intestinal fluid. It is prerequisite for these drugs to be absorbed and transported in native conformation and structures for the physiological activity. Furthermore when they are formulated in combination, the stability profiles in such fluid need to be elaborated for the predication of physiological activity at target site[15, 16].

The activity of proteins depends on the three-dimensional molecular structure. The dosage form development of proteins may expose the proteins to harsh conditions that may alter their structure. When these incompabilities proceed with excipients they will have implications in the efficacy and immunogenic response. The excipients induced incompatibility and consequent physical degradation involves modification of the native structure of a protein to a higher-order structure, which may be a result of adsorption, aggregation, unfolding, or precipitation. Sometimes these incompabilities may induce chemical degradation which usually involves bond cleavage and leads to the formation of a new product. Chemical degradation is preceded by a physical process such as unfolding, which exposes the hidden residues to chemical reactions. The processes involved in chemical degradation are deamidation, oxidation, disulfide exchange, and hydrolysis. Proteins must be characterized for change in conformation, size, shape, surface properties, and bioactivity upon formulation processing. Further the intestinal luminal content induced change in pH may also alter the isoelectric points of native proteins and subsequently the solubility of proteins may be affected[16, 17].

MATERIALS AND METHOD

Serratiopeptidase or serrapeptase (MW 52 kDa) was supplied as a gift by twilight litaka pharma (Mumbai). Bovine serum albumin was purchased from Loba chemicals (Mumbai, India).Casein, L-Tyrosine, trichloroacetic acid (TCA) and folin's ciocalteau reagent were obtained from Sd fine chemicals Ltd (Mumbai, India). Reagents used in peptide assay were of analytical grade. All other chemicals and glassware used in study were supplied by store department Gurunanak College of pharmacy, Nagpur. All experiments on animal studies were performed with due permission from animal ethical committee.

Feasibility

The preliminary screening was executed for the consideration of serratiopeptidase for innovative microparticles preparation. The feasibility studies with the above peptide include the development of solvent systems, solubility, isoelectric point consideration,

compatibility with the incorporated excipients and stability.

Preparation of Microspheres
Microparticles were prepared by solubilising 3 gm of albumin in 100 ml of water. About 120 ml of acetone were added to this solution in order to coacervate the albumin. Acetone was evaporated by heating the coacervate at 65°C. Thus obtaining cross linked albumin microspheres. The suspension was finally dried. The optimization includes the variants of albumin, water to acetone ratios. The acetone can also be replaced by dichloromethane and methanol or mixture of both. An aliquot of 50 mg of microparticles was then transferred to 25 ml of an aqueous serrapeptase solution (1 mg/ml) and left under agitation for 24 hours. The loaded microparticles were separated from remaining solution by centrifugation and filtration. Microparticles was then washed with water and filtered again. Microparticles coating was carried out as follows: 300 mg of microparticles were transferred into a beaker containing 50 ml of an organic solvent blend consisting of methanol, acetone, and dichloromethane solution of 250 mg of eudragit S-100. The suspension obtained was kept under agitation until the organic solvent was evaporated. Finally the enteric coated microsphere was then washed, filtrated and dried.

Characterization of the Microspheres
Encapsulation Efficiency
Twenty milligrams of microspheres were accurately weighed. They were added to 5 ml of ethanol. After the microspheres dissolved completely, 5 ml of phosphate buffer (pH 7.4) was added to this solution and mixed thoroughly. The resulting solution was analyzed for SRP content by UV-spectroscopic method (Shimadzu UV A-1700, Pharmaspec, Tokyo, Japan), using phosphate buffer (pH 7.4) and ethanol mixture (1:1) as blank. Encapsulation efficiency (%) was calculated using the following formula[18].

$$\text{Actual Loading} = \frac{\text{Mg of encapsulated SRP}}{\text{100 mg of microspheres}}$$

$$\text{Encapsulation Efficiency} = \frac{\text{Actual SRP loading}}{\text{Theoretical SRP loading}} \times 100$$

Particle Size
Particle size analysis of peptide-loaded Eudragit S100 microspheres was performed by optical microscopy using a Motic microscope[19]. A small amount of dry microspheres was suspended in purified water (10mL). The suspension was ultrasonicated for 5 seconds. A small drop of suspension thus obtained was placed on a clean glass slide. The slide containing microspheres was mounted on the stage of the microscope and 300 particles were measured using a calibrated ocular micrometer. The process was repeated for each batch prepared.

Morphology
The surface morphology and shape of the microspheres were analyzed by scanning electron microscopy for selected batches (Leo, VP-435, Cambridge, UK). Photomicrographs were observed at ×200 magnification operated with an acceleration voltage of 15 kV and working distance of 19 mm was maintained. Microspheres were mounted on the standard specimen-mounting stubs and were coated with a thin layer (20 nm) of gold by a sputter-coater unit (VG Microtech, Uckfield, UK).

Drug Content[18]
Twenty milligrams of the dried microspheres were accurately weighed. They were added to 5 mL of ethanol. After the microspheres dissolved completely, 5 mL of phosphate buffer (pH 7.4) was added to this solution and mixed thoroughly. The resulting solution was filtered using a Whattman filter (0.45-μm pore size) and analyzed for SRP content by UV-spectroscopic method (Shimadzu UV A-1700,

Pharmaspec, Tokyo, Japan) as described earlier.

In-Vitro Proteolytic Activity[21]

Prepared Eudragit S-100 microspheres and plain SRP solution were placed separately in 5 ml of HCl buffer (pH 1.2) and phosphate buffer (pH 7.4) maintained at 37 ± 0.5°C and stirred constantly at 100 rpm. After 2 hours, samples were recovered by centrifugation and filtration at room temperature (n = 3). The peptide activity was then estimated in acidic and basic environment by UV-spectroscopic method.

***In-Vitro* Drug Release[20]**

***In-vitro* Release in Phosphate Buffer pH 7.4**

In vitro release of SRP from microspheres was evaluated in phosphate buffer (pH 7.4). Microspheres equivalent to 20 000 U of SRP were transferred to the prewarmed dissolution media (20 mL) and maintained at 37°C ± 0.5°C under stirring at 50 rpm. Samples were withdrawn every hour up to 12 hours, and the volume was replaced immediately by fresh phosphate buffer. The sample withdrawn was centrifuged and filtered. SRP content of the supernatant was estimated by Spectrophotometric method, against a phosphate buffer (pH 7.4) blank[22].

***In-vitro* Drug Release with Rat Caecal Content**

Due to the similarity of human intestinal microflora with the rat intestinal contents, the drug release studies were carried out in presence of caecal contents and its effect on the in-vitro release profiles of serratiopeptidase was studied. The colonic caecal contents were prepared as follows: Thirty minutes before commencement of studies, five rats were killed by euthanasia. Their abdomen were opened, the caecam were isolated ligated at both the ends, dissected and immediately transfer into pH 7.4. The caecal bags were opened, their contents were individually weight pooled and then suspended in buffer to give final dilution of 4 % w/v[23]. Further pH of the solution was maintained to 6.8 with phosphate buffer. Microspheres equivalent to 20 000 U of SRP were transferred to the prewarmed dissolution media (20 mL) and maintained at 37°C ± 0.5°C under stirring at 50 rpm. Samples were withdrawn every hour up to 12 hours, and the volume was replaced immediately by fresh phosphate buffer. The sample withdrawn was centrifuged and filtered. SRP content of the supernatant was estimated by Spectrophotometric method, against a phosphate buffer (pH 7.4) as blank.

RESULTS AND DISCUSSION

The preliminary studies show that albumin and Eudragit were compatible with SRP. The feasibility studies revels that pH 9 will be suitable for drug entrapment as optimum activity SRP was observed at this pH. The temperature suitable for most of formulation processing was found to be 40°c. The isoelectric point (pI) of Serratiopeptidase is 6.1and if the pH of solution is far from the pI, the difference in the apparent pH and actual pH may not have a large effect on the stability or conformation of protein. This suggests the possibility of maximum activity at pH 9. Even if these have been correlated with the literature suggest that pH values equidistantly above and below the pI should not be assume to afford equivalent and/ or linear increase in solubility.

Characterization of Microparticles

Encapsulation efficiency and drug content

The amount of peptide entrapped was calculated. It has been found that during the preparation of microparticles the changes in organic solvent ratio changes encapsulation efficiency. Replacement of acetone with mixture dichloromethane (DCM), ethyl acetate (EA) and methanol (ME) increases encapsulation efficiency. Peptides are highly hydrophilic in nature in due they will be loss if proportion of quite polar solvents is increases. In the first step entrapment the semipermiable membrane of albumin governs the entrapment. Once entrap the high molecular size of peptide refuses its loss in water. Finally at the coating process it will be liable to be come out of albumin microparticles. Where increment in DCM: ME: EA ratio increases entrapment and drug content all together. The parameters are shown in Table 1.

Table 1: Formulation Parameters of Microparticles

Batch Code	Production Yield %	Theoretical Drug Content %	Actual Drug Content %	Drug Entrapment Efficiency (E.E)%
PFX-1	84.17±1.35	550	39.99±0.23	49.98±1.07
PFX-1	88.48±1.23	33.33	11.75±0.67	67.25±1.12
PFX-1	74.59±1.86	35	21.24±0.55	78.96±1.95
PFX-1	78.98±1.97	30	19.95±0.30	79.75±1.34
PFX-1	70.72±2.01	19.66	12.88±0.47	87.30±2.01
PFX-1	60.09±1.52	34.31	32.17±0.38	98.51±1.05

Morphology and Particle Size

The scanning electron microscopy conform the spherical nature of the microparticles. The most particles with rounded surface morphology reveal the spherical nature of microparticles. Figure 1a and 1b represents intact morphology and nature of drug release with incubation in caecal contents.

Particle size of the microparticles was studies using optical Motic microscope and scanning electron microscopy. The optimization study during initial phase of phase separation-coacervation reveals that increase in the proportion of acetone decreases the final size of the microparticles. Even during the initial coating process optimization, it has been found that the final size of microparticles depends upon the coacervation process. The most of optimized microparticles was found to be in the range of 140-150 µm. Figure 2 shows particles size distribution during particles size measurement.

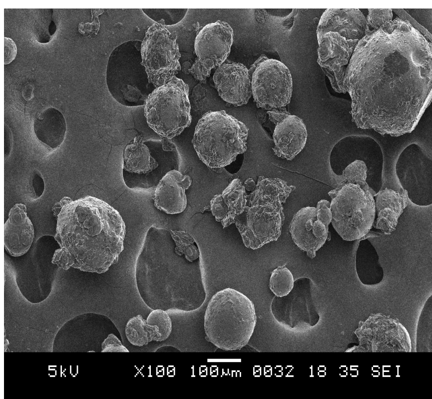

Fig. 1a: Scanning Electron Micrograph of Intact Particles

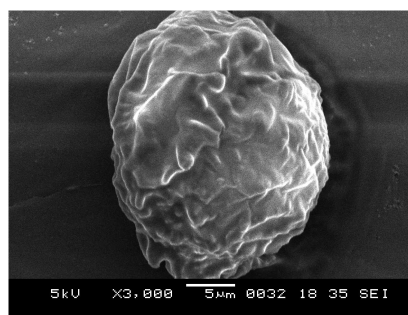

Fig. 1b: Scanning Electron Micrograph of Microparticles with Incubation in Caecal Contents

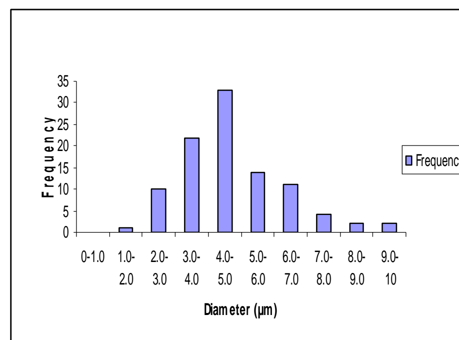

Fig. 2: Particle Size Distribution

In-vitro Proteolytic Activity

Drug entrapped in microparticles shows only 0.3 % loss of Proteolytic activity than intact serratiopeptidase with 98% loss of activity in acidic medium. In HCl, Eudragit S-100 found to prevent loss of activity, while with phosphate buffer (pH 7.4) 37 ± 0.5°C, 98% of activity will be release in dissolution media. The Proteolytic activity studies show intact nature of microparticles for prevention of loss of activity against acidic media.

In-vitro Drug Release

The *in-vitro* serrapeptase release study was performed according to USP-XXIII method. The drug release characteristics were studied to access the delivery of SRP in intestine. The typical release curves were obtained with phosphate buffer and caecal contents was shown in figure 3 and 4 respectively. The initial lag phase delays the release up to five hours followed by complete release over the next eight hours. The scanning electron microscopic photograph with incubation in colonic caecal contents shows drug release by diffusion.

The numbers of batches were studied in presence of colonic caecal contents to access any change in release characteristic of microparticles. The study reveals there is almost no change in release properties of drug in caecal contents. The release was found to follows Fickian diffusion mechanism.

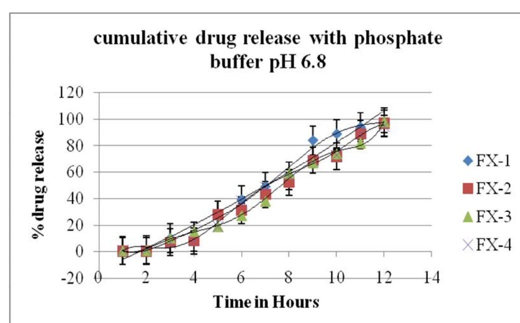

Fig. 3: Cumulative Drug Release with Phosphate Buffer

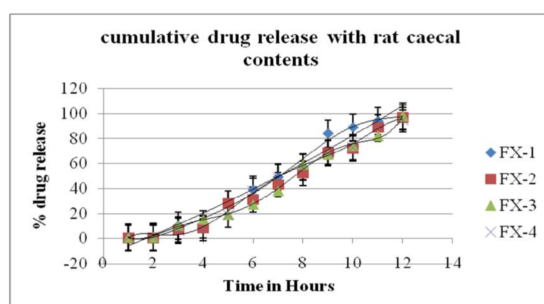

Fig. 4: Cumulative Drug Releases with Rat Caecal Contents

CONCLUSION

Serratiopeptidase entrapped enteric coated crossed linked albumin microspheres retard the release of SRP at low pH and release slowly at pH 7.4 in the colon. Drug release study with colonic caecal contents demonstrated a prominent serratiopeptidase release without any loss in activity, suggesting that the polymer could protect SRP against Proteolytic degradation in the GI tract. Eudragit S-100 microspheres, thus, have the potential to serve as an oral carrier for peptide drugs like Serratiopeptidase. These microspheres are able to entrap the peptide at high levels where the inner albumin core containing the drug would allows kinetic control of release and coating ensures drug core localization in intestinal tract. Enteric coated cross linked albumin microspheres can be considered as promising delivery systems for oral delivery of peptide drugs like serratiopeptidase.

REFERENCES

1. Woodley JF. Enzymatic Barriers for GI Peptide and Protein Delivery. Crit Rev Ther Drug Carrier Syst. 1994;11:61–95.
2. Swarbrick J and Boylan JC. Encyclopedia of Pharmaceutical Technology. 2nd ed., New York: Marcel Dekker, Inc. 2002;885.
3. Ashford M, Fell JT. Targeting drugs to the colon. Delivery Systems for Oral Administration. J Drug Target. 1994;2:241-258.
4. Yamamoto A, Taniguchi T, Rikyun K, Tsuji T, Fujita T, Murakami M and Muranishi S. Effects of Various Protease Inhibitors on the Intestinal Absorption and Degradation of Insulin in Rats. Pharm Res. 1994;11:1496–1500.
5. Krauland AH, Guggi D and Bernkop SA. Oral insulin delivery. The potential of Thiolated Chitosan-Insulin Tablets on Non-Diabetic Rats. J Control Rel.2004;95: 547–555.
6. Cui FD, Tao AJ, Cun DM, Zhang LQ and Shi K. Preparation of Insulin Loaded PLGA-Hp55 Nanoparticles for Oral Delivery. J Pharm Sci. 2007;96:421–427.
7. Hinton JM, Lennard JJE, Young AC. A New Method for Studying Gut Transit Times Using Radioopaque Markers. Gut. 1969;10:842.
8. Johnson RE, Lanaski LA, Gupta V, Griffin MJ, Gaud HT, Needham TE and Zia H. Stability of Atriopeptin III in Poly (DL-lactide-*co* glycolide) Microspheres. J Controlled Release. 1991;17:61–68.

9. Soriano I, Evora C and Llabres M. Preparation and Evaluation of Insulin-Loaded Poly (DL-lactide) Microspheres Using an Experimental Design. Int J Pharm. 1991;142:135–142.
10. Calis S, Jeyanthi R, Tsai T, Mehta RC and Deluca PP. Adsorption of Salmon Calcitonin to Plga Microspheres. Pharm Res.1995;12:1072–1076.
11. Tsai T, Mehta RC and Deluca PP. Adsorption of Peptides to Poly (DL-lactide-*co*-glycolide). Effect of Physical Factors on the Adsorption. J Controlled Release. 1996a;127:31–42.
12. Tsai T, Mehta RC and Deluca PP. Adsorption of Peptides to Poly(DL-lactide-*co*-glycolide). Effect of Solution Properties on the Adsorption. J Controlled Release 1996b;127:43–52.
13. Ishibashi T, Hatano H, Yoshino H, Mizobe M and Kobayashi M. Int J Pharm in press.
14. Davis SS. The design and Evaluation of Controlled Release Systems for the Gastrointestinal Tract. J Control Release. 1985;2:27–38.
15. Swarbrick J and Boylan J. Encyclopedia of Pharmaceutical Technology.2nd ed.; (Marcel Dekker, NY). 2002;885.
16. Ashford M and Fell J. Targeting Drugs to the Colon. Delivery Systems for Oral Administration. J Drug Target.1994;2:241-258.
17. Yamamoto A, Taniguchi T, Rikyun K, Tsuji T, Fujita T, Murakami M and Muranishi S. Effects of Various Protease Inhibitors on the Intestinal Absorption and Degradation of Insulin in Rats. Pharm Res.1994;11:1496–1500.
18. Jayvadan K, Pate1 and Jayant RC. Formulation and Evaluation of Stomach-Specific Amoxicillin Loaded Carbopol-934P Mucoadhesive Microspheres for Anti-Helicobacter Pylori Therapy. Journal of Microencapsulation. 2009;26(4):365–376.
19. Dashora K and Saraf S. Effect of Processing Variables on Micro Particulate System of Aceclofenac. Pak J Pharm Sci. 2006;19:610.
20. United States Pharmacopoeia/ National Formulary 2007.
21. Indian Pharmacopoeia, Central Indian Pharmacopeia Laboratory, Govt. of India, Ministry of Health and Family Welfare. 2010;76-135.
22. Indian pharmacopoeia, V.H. Govt. of India, Ministry of Health and Family Welfare 1996;189.
23. Ramprasad V, Krishnarah S and Satynarayan S. J of Controlled Release.1998;51:281.