



ISSN: 0975-766X  
CODEN: IJPTFI  
Research Article

Available Online through  
www.ijptonline.com

**THE INTEGRITY OF PROTEIN ASSOCIATED WITH ENTERIC COATED TABLET  
EXCIPIENT IN FORMULATION AND ITS DEGRADATION IN SIMULATED  
INTESTINAL FLUID**

Mitali M. Bodhankar<sup>1</sup>, Vinit V. Agnihotri<sup>1\*</sup> and Bhushan Bhojar<sup>1</sup>

<sup>1</sup>Gurunank College of Pharmacy,  
Nari Road, Near Dixit Nagar, Nagpur-440 026.  
Email: vinitagnihotri\_05@rediffmail.com

Received on 21-06-2011

Accepted on 06-07-2011

**Abstract:**

The excipients induced instability in protein and peptide drug may follow the physical and chemical degradation of native protein. Serratiopeptidase (SRP) was used as a model peptide. SRP was studied for *in-vitro* degradation characteristics with enteric polymeric excipients such as eudragit S-100. Assessment and type of degradation was analysed by Spectrophotometry, kinetic mode UV spectroscopy and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The above investigation reveals that the model protein follows degradation with eudragit S-100 in SIF with no new fragments. Therefore it may be concluded that when such peptides are formulated with enteric excipients the aggregation follows with adsorption on enteric polymers in simulated intestinal fluid.

**Keywords:** Serratiopeptidase, Eudragit S-100, SDS-PAGE, Spectrophotometry.

**Introduction**

Protein and peptides are important class of potent therapeutic drugs. However their susceptibility to chemical and physical degradation makes formulation and development more challenging than conventional drugs. This is in

part due to incompatibilities with excipient and unique physicochemical and biological properties of proteins and peptide drugs<sup>1,2</sup>.

The excipient induced instability in protein and peptide drug may follow the physical and chemical degradation of native protein. These incompatibilities 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 incompatibilities 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<sup>2,3</sup>.

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 incompatibilities proceed with excipient they will have implications in the efficacy and immunogenic response. The excipient 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 incompatibilities 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 excipients induced change in pH may also alter the isoelectric points of native proteins and subsequently the solubility of proteins may be affected<sup>3,4</sup>.

The present study is design to investigate the stability profile of peptide drugs subjected to the oral administration and formulated with enteric coated tableted excipient. The stability of peptides is investigated in simulated intestinal fluid with their co-manufactured enteric excipients<sup>4,5</sup>.

### **Materials and Methods:**

Commercial SRP enteric film coated tablets (10 mg, molecular weight 52 kDa; Biosuganril\*10, Piramal healthcare) and Eudragit S100 (Zim laboratories, Nagpur). Sodium dodecyl sulphates (SDS), monobasic potassium phosphate, sodium hydroxide (NaOH) were obtained from pharmaceutics laboratory, Gurunanak college of pharmacy, Nagpur. Low molecular weight markers for protein analysis were provided by Hislop college, Nagpur. The SDS-PAGE analysis was performed at Hislop college, Nagpur. The spectrophotometric analysis was done on shimazdu A-1700, incubator used is orbital mechanical shaker and incubator (REMI).

### **Preparation of simulated intestinal fluid:**

Accurately weight quantity of monobasic potassium phosphate (6.8g) in 250 ml of water was mix and dissolved. Sodium hydroxide (0.2 N, 77ml) was then added with 500 ml of water. To the above solution pancreatin (10g) was added and mixed properly. The resulting solution was adjusted to the pH  $6.8\pm 0.1$  with 0.2 N NaOH or 0.2 N HCl and volume was made to 1000ml.

### ***In-Vitro* degradation of peptide enteric coated film coated tablets:**

#### ***Group Ia-Enteric coated peptide in simulated intestinal fluid (SIF) with Incubation:***

The enteric tablet samples were placed in simulated intestinal fluid (SIF) prepared from the procedure as given in section 3. They were incubated in orbital shaker incubator at 37°C. They were retained in separate tubes for 8 hours. The samples were removed every hour and analysed for degradation if any by spectrophotometry and electrophoresis.

***Group Ib-STD peptide in with Incubation:*** The STD peptide was dissolved in DW to access the change if any occurred through the contact of water only. They were incubated in orbital shaker and incubator at 37°C. They

were retained in separate tubes for 8 hours. The samples were removed every hour and analysed for degradation if any by spectrophotometry and electrophoresis.

***Group IIa-Enteric coated peptide in SIF without incubation:***

The samples of given enteric tablets were placed in SIF (prepared as given in section 3) at room temperature, without incubation. The samples were removed every hour and analysed for degradation if any by spectrophotometry and electrophoresis.

***Group IIb-STD peptide without Incubation:***

The STD peptide was placed in DW at room temperature, without incubation. The samples were removed every hour and analysed for degradation if any by spectrophotometry and electrophoresis.

**Determination of protein structural integrity with enteric excipient by SDS-PAGE:**

The raw samples were obtained from given group with released protein and associated enteric excipient. The excipient may interfere with protein integrity analysis, as both are high molecular weight component. The protein was extracted from given sample with removed excipient and then analysed for SDS-PAGE.

***Preparation of samples for electrophoresis:***

The raw samples as collected from given group Ia, Ib, IIa, IIb, were centrifuged. The supernatant were separated and SDS- PAGE sample buffer was added. The samples were then resuspended in 0.25 ml of 5% w/v SDS solution. A mixture of dichloromethane 0.5ml (DCM) and acetone 0.5 ml was added to dissolve the enteric polymer. The organic phase was removed and samples were subjected to SDS-PAGE analysis.

***Preparation of solutions for SDS-PAGE:***

1. Solution A was prepared by accurately weighing 30g of acrylamide and 0.8g of bisacrylamide and was dissolved in 100ml of water. Solution B is prepared by accurately weighing 18.66g tris-HCl and dissolved in 100ml distilled water, pH 8.8. Solution C was prepared by accurately weighing 6.55g tris-HCl in 100ml of distilled water, pH 6.8. 10% SDS, 10g is dissolved in 100 ml distilled water. 10% 2-mercaptoethanol solution was prepared and 1 ml of this solution is diluted up to 10ml distilled water.

2. Tracking dye was prepared by mixing 2ml of above solution C with 1.5ml 10% SDS. 2ml of glycerol is added to it. Further 1ml 2-mercaptoethanol was mixed and 25mg bromophenol blue was dissolved with it.
3. Tracking buffer was prepared as 0.5g tris buffer with 14.4g glycine was mixed with 1g SDS in 1ml distilled water, pH-8.3.
4. Coomassie blue 0.2% w/v in methanol:acetic acid:water in the ratio 4:1:5, The above solution was filtered and store in dark bottle.
5. De-staining solution comprise of methanol:acetic acid:water in ration 4:1:5.

***Processing of prepared samples by SDS-PAGE:***

Sodium dodecyl sulphate is a detergent that readily binds to proteins. At pH-7 in the presence of 1% w/v SDS and 2-mercaptoethanol, proteins dissociate into their subunits and bind about 1.4g of SDS per gram of protein which completely masks the natural charge of the protein giving a constant charge to mass ratio. The larger the molecule therefore the greater the charge so the electrophoretic mobility of the complex depends on the size (mol. wt.) of the protein and a plot of log mol wt. against relative mobility gives a straight line. The molecular wt. of a protein is determined by comparing its mobility with a series of protein standards. The sieving effect of the polyacrylamide is important in this technique and the range of molecular wt that can be separated on a particular gel depends on the pore size of the gel. The amount of crosslinking and hence pore size in a gel can be varied by simply altering the amount of acrylamide to make 5% or 10%.

The protein (52 kDa) extracted with procedure as given in section 5-I and molecular weight markers solubilised with sample buffer were loaded onto a vertical slab gel and subjected to electrophoresis at 50 V till the tracking dye reached the boundary of stacking and running gel. As the tracking dye entered running gel, the voltage was increased to 100 volts and when it reached 3/4 of the gel, electrophoresis was stopped. The gel was carefully removed from the glass slabs and proteins were visualised by staining overnight with coomassie brilliant blue reagent (0.1% w/v) in water:methanol:acetic acid (4:5:1) and destained overnight in

water:methanol:aceticacid(4:5:1).

### **Determination of protein degradation with enteric excipient by spectrophotometry.**

The raw samples as collected from given group Ia, Ib, IIa, IIb, were centrifuged. The supernant were separated and then resuspended in a mixture of DCM (0.5ml) and acetone (0.5 ml) to dissolve the enteric polymer. The above solution was then filtered and subjected UV-analysis.

### ***Spectral analysis of protein associated with enteric excipient.***

The shift in absorption maxima and change in spectral (area) movement was studied for all the samples from given group Ia, Ib, IIa, IIb. All the samples were pre-treated as mentioned earlier. The shimazdu A-1700 spectrophotomer was used analysed the above samples in spectrum mode.

### **Stability analysis of protein associated with enteric excipient in kinetic mode:**

The stability of given protein samples with enteric excipients were studied. The samples were removed at 2, 4, 6, 8 hour interval and pre-treated as given in section 6. All the samples of known concentration (10mg) associated with enteric excipient were subjected to two hour kinetic mode analysis in shimazdu A-1700 spectrophotometer. The changes in absorbance were recorded for above samples from all the groups.

## **Results and Discussions**

### **Spectral analysis Kinetic mode UV spectroscopy:**

The spectral study shown that the above STD peptide shows two absorption peaks. In part the first peak is of SRP having absorption maximum at 229.5 nm. The absorption maximum at 265 nm was may be due to the peptide linkage of the model peptide. Following incubation of given protein with enteric excipient it has been observed that peak at 265 nm gets broaden edifying the change in protein component of sample. The change is found to be progressive with the incubation (fig.1). SRP is quite stable compared to other peptide usually given parenterally. The STD peptide in distilled water shows that there is no change in spectral characteristic of the peptide at both absorption maxima either incubated or not (fig: 2 & 3). Therefore, it may be interpreted that incubation and STD peptide in distilled water would not have any impact on the stability of peptide.

Fig: 1 Spectral analysis of Peptide with excipients (A) and STD peptide (B).

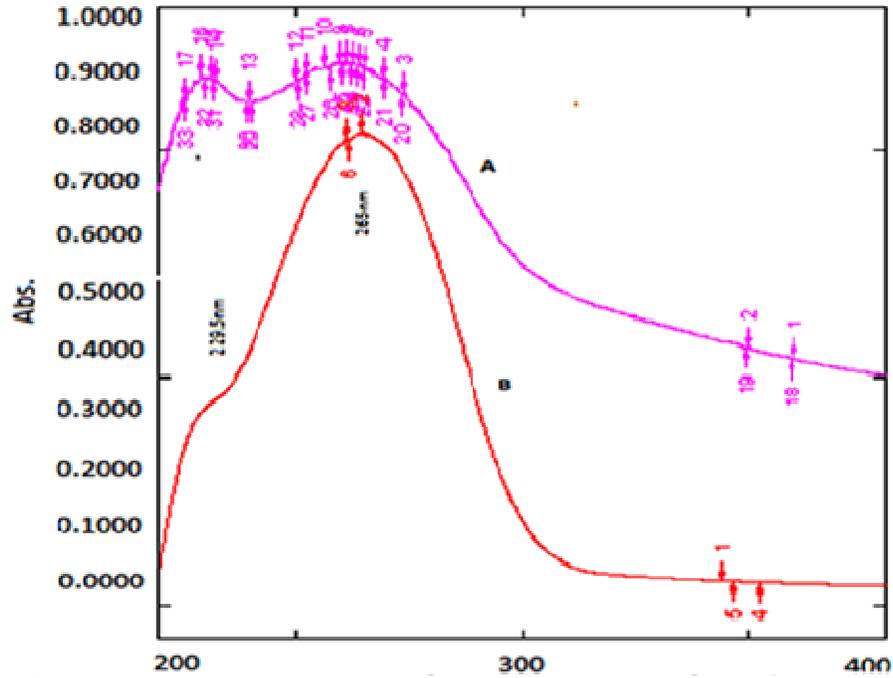
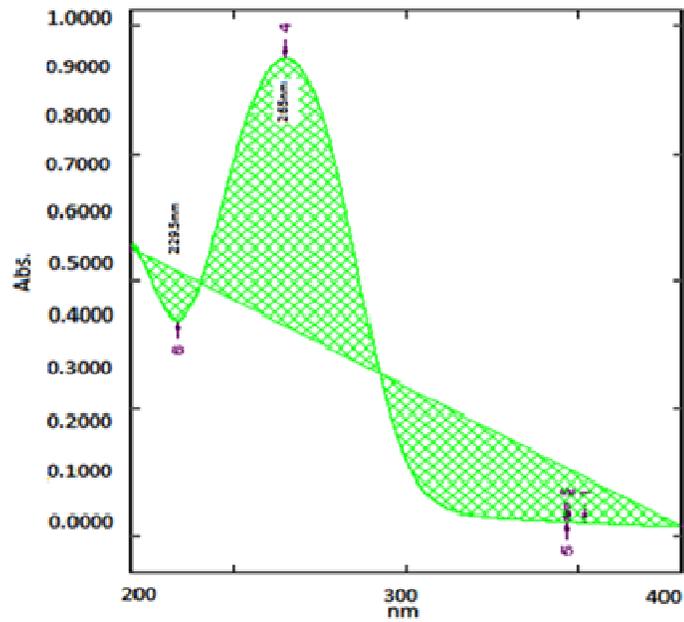
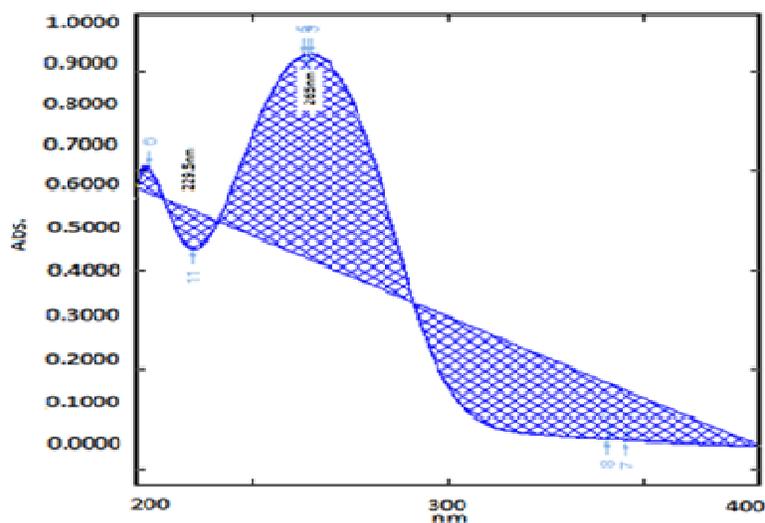


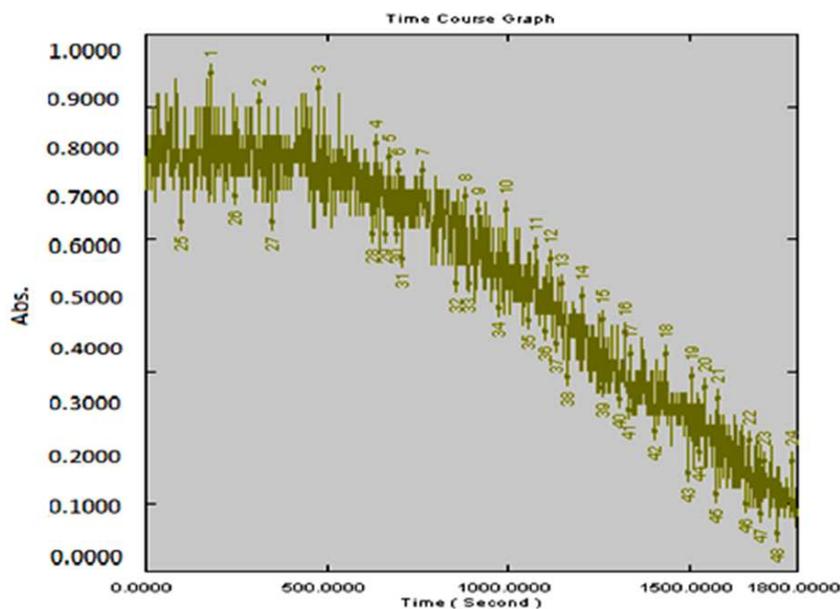
Fig: 2 STD peptide without incubation.



**Fig: 3 STD peptide with incubation.**



**Fig: 4 Kinetic mode Spectroscopy of peptide with enteric excipients.**



When it is incubated in SIF with enteric excipient it shows definite change in the spectral characteristic of peptide component (Fig.1). There may be possibility that some aggregation is initiated due to which the peak at 265 nm gets broaden.

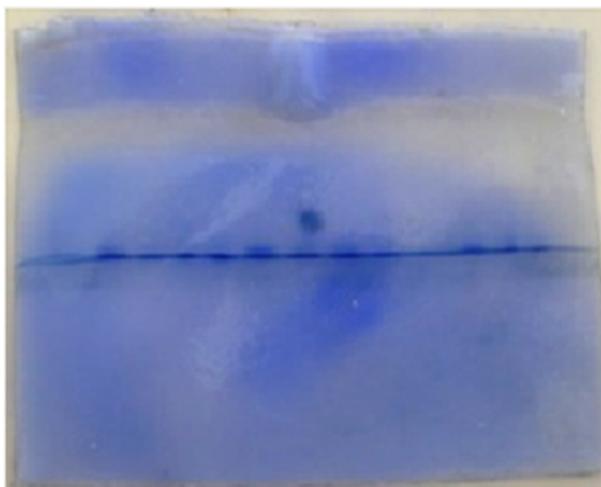
The stability study was carried out for two hour in kinetic mode of the Shimadzu UV A-1700 spectrophotometer. The data obtained is shown in fig 4. The observation revealed that the absorbance is decreasing of native peptide with proceeding time. From fig.4, the degradation is initiated after initial 500 second. Therefore the peptide coated in enteric polymers may proceeds degradation SIF.

#### **Electrophoresis studies:**

Following the electrophoresis using SDS-PAGE it has been observed that no single lane diverted from the normal. The lanes travel the same distance (52kDa) for all the groups signifying that there is no molecular weight variation. Therefore there may be no new substances are form. Even traces of other component are not found with all applied procedure for STD peptide in DW and peptide with excipients in SIF and DW (Fig 5).

The peptides drug generally have primary, secondary and tertiary structures, unlike conventional drugs and in solution may adopt several different conformations depending upon their size. It is prime requisite to preserve the pharmacologically active conformation during the process of formulation<sup>6,7</sup>. But still for physiological action it should not also change conformation in gastric/intestinal fluid. Even, when they are incorporated with enteric excipient which allows the drug to release at pH-7, necessitates the through studies on the ionization of peptide and isoelectric point characteristics<sup>7, 8 and9</sup>.

**Fig: 5 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis analysis.**



**Lane I to V: Group Ia, Ib, IIa, IIb, STD peptide**

At isoelectric point the aqueous solubility of peptide is minimal where the drug is neutral or has no net charge. Excipient like reducing sugars can react with protein amino group to form Schiff's base<sup>10, 11, and 12</sup>. When the enteric excipients are incorporated the pH induced unfolding leads to state of lower electrostatic free energy<sup>13, 14</sup> and<sup>15</sup>. When protein possesses both positive and negative charges, anisotropic charge distribution leads to dipole. In this case, repulsive protein-protein interaction could make assembly processes such as aggregation energetically favourable. This may be the rationale why degradation was proceeds in this case without any new component formed. It may also be possibility that peptide would be absorbed on the eudragit S-100 when it was place in DW and released during incubation period (fig: 1). These studies are important when the dosage form is allowed to remain in intestinal tract for longer duration of time eg. Sustained delivery of biotechnologically derived product. The conformation of protein should remain the same throughout the absorption and transit time.

## Conclusion

When increase gastric residence of dosage form is required (eg. Sustained oral delivery) the stability studies are essential in bio-phase environment to target the site with native peptide structure and specificity. The above investigation reveals that the model protein follows degradation with eudragit S-100 in SIF with no new fragments. Therefore it may be concluded that when such peptides are formulated with enteric excipients the aggregation follows with adsorption on enteric polymers in simulated intestinal fluid. The compatibility studies with the excipient should be carried in biological fluids out for such biotechnologically derived product for efficient delivery at target site.

## References

1. Woodley J. Enzymatic barriers for GI peptide and protein delivery. *Crit Rev Ther Drug Carrier Syst.* 1994; 11: 61–95.
2. Swarbrick J, Boylan J, editors. *Encyclopedia of pharmaceutical technology.* 2nd ed., (Marcel Dekker, NY, 2002) 885.

3. Ashford M, Fell J. 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, 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 A, Guggi D, Bernkop-Schnürch A. Oral insulin delivery: the potential of thiolated chitosan-insulin tablets on non-diabetic rats. *J. Control Rel*. 2004; 95: 547–555.
6. Cui F, Tao A, Cun D, Zhang L, Shi K. Preparation of insulin loaded PLGA-Hp55 nanoparticles for oral delivery. *J Pharm Sci*. 2007; 96: 421–427.
7. Hinton J, Lennard-Jonnes, J, Young A. A new method for studying gut transit times using radioopaque markers. *Gut*, 1969; 10: 842.
8. Morishita M, Peppas N. Is the oral route possible for peptide and protein drug delivery? *Drug Discov. Today*, 2006; 11: 905 – 910.
9. Almeida A, Souto E. Solid lipid nanoparticles as a drug delivery system for peptides and proteins . *Adv. Drug Del. Rev.*, 2007; 59: 478 – 490.
10. Pearlman R, Nguyen T. “Analysis of protein drugs,” In peptide and protein drug delivery, V.H. L. Lee, Ed. (Marcel Dekker, NY, 1991) Chapter 6.
11. Hoffmann H. “Analytical methods and stability testing of biopharmaceuticals,” In Protein formulation and delivery, E.J.McNally, Ed. (Marcel Dekker, NY, 2000) Chapter 3.
12. Tozaki H. et al., “Degradation of insulin and calcitonin and their protection by various protease inhibitors in rat caecal contents: implications in peptide delivery to the colon,” *J. Pharm. Pharmacol*. 1997; 49: 164–168.
13. Marschutz M, Bernkop-Schnurch A. “Oral peptide drug delivery: polymer- inhibitor conjugates protecting insulin from enzymatic degradation in vitro,” *Biomaterials* 2000; 21: 1499–1507.
14. Semalty A., Semalty M., Singh R., Saraf S., Saraf S. Properties and formulation of oral delivery system of protein and peptides, *Indian j. pharm. Sci*. 2007, 69 (6): 741-747.

15. Jacob S., Shirwaikar A., Srinivasan K., Alex J., Prabhu S., Mahalxmi R., Kumar R. Stability of proteins in aqueous solution and solid state, *Indian j. pharm. Sci.* 2006, 68 (2): 154-163.

**Corresponding Author:**

**Vinit V. Agnihotri\***,

**Email:** [vinitagnihotri\\_05@rediffmail.com](mailto:vinitagnihotri_05@rediffmail.com)